RNA-RNA interactions in the process of 70 S initiation complex formation between E. coli ribosomes and bacteriophage Qβ RNA

INAUGURAL-DISSERTATION

ZUR

ERLANGUNG DER PHILOSOPHISCHEN DOKTORWÜRDE

VORGELEGT DER

PHILOSOPHISCHEN FAKULTÄT II

DER

UNIVERSITÄT ZÜRICH

VON

TADATSUGU TANIGUCHI

AUS JAPAN

BEGUTACHTET VON HERRN

PROF. Dr. C. WEISSMANN

1979

ZÜRICH 1978

Sinnihon Insatsu Co. L., Tokyo

printed by Sinnihon Insatsu Co. L., Tokyo, Japan 1979

RNA-RNA interactions in the process of 70 S initiation complex formation between E. coli ribosomes and bacteriophage Q β RNA

INAUGURAL-DISSERTATION

ZUR

ERLANGUNG DER PHILOSOPHISCHEN DOKTORWÜRDE

VORGELEGT DER

PHILOSOPHISCHEN FAKULTÄT II

DER

UNIVERSITÄT ZÜRICH

VON

TADATSUGU TANIGUCHI

AUS JAPAN

BEGUTACHTET VON HERRN

PROF. Dr. C. WEISSMANN

ZÜRICH 1978

Sinnihon Insatsu Co. L., Tokyo

UNIV.
LEIDEN
BIBL.

To my parents

To Yoko

Acknowledgments

I wish to express my gratitude to Prof. Dr. C. Weissmann. This thesis would not have been possible without his continuous advice, support and encouragement during my stay in Zürich.

I also thank all the collaborators in the institute, especially Prof. Dr. M. Billeter, Dr. H. Weber, Dr. D. Sabo (†), Dr. E. Domingo, Dr. C. Escarmis, Dr. A. Shapira and Dr. F. Meyer for valuable discussions and advice.

Thanks are also due to Ms. R.M. von Rotz, Ms. A. Schmid, Mr. T. Weibel, Ms. L. Boller and Ms. F. Christen for the help they kindly offered to me.

I am grateful to Dr. S. Nagata for the critical reading of the thesis as well as for valuable advice.

I also wish to thank Prof. Dr. M. Libonati (University of Naples) and Prof. Dr. T. Ikawa (University of Tsukuba) who introduced me to this field and gave me continuous moral support.

C O N T E N T S

INTRODUCTION

RESULTS

Section I: Interaction of E.coli ribosomes with Qβ RNA

(A) Binding of ribosomes to Qβ RNA in the presence and in the absence of fMet-tRNA

(B) The structure of oligonucleotides P-2a, P-2b, P-4 and P-15

(C) Interaction of oligonucleotide P-2a with the 3' end of 16 S rRNA within the 30 S ribosome

Section II: Inhibition of Qβ RNA 70 S ribosome initiation complex formation by an oligonucleotide complementary to the 3' terminal region of E.coli 16 S ribosomal RNA

Section III: Site-directed mutations in the initiator region of the Qβ coat cistron and their effect on ribosome binding

(A) Insertion of N^4-hydroxyCMP into the Qβ minus strand positions complementary to the third and fourth nucleotide of the coat cistron

(B) Analysis of plus strands synthesized with substituted minus strands as template

a) Isolation and analysis of the replicase binding site "S"

b) Analysis by two-dimensional polyacrylamide gel electrophoresis

(C) Ribosome binding capacity of Qβ RNA with G → A transitions in the third and fourth nucleotides of the coat cistron

DISCUSSION

(A) Interaction of ribosomes with Qβ RNA in the presence and in the absence of fMet-tRNA

(B) Importance of 16 S rRNA 3' terminal region in the formation of 70 S initiation complexes between ribosomes and Qβ RNA

(C) Role of initiator A-U-G in the 70 S initiation complex formation

(D) RNA-RNA interactions in the ribosome binding process

(E) Role of secondary (or tertiary) structure of mRNA in the ribosome binding process

(F) Involvement of proteins in the ribosome binding process

SUMMARY

MATERIALS AND METHODS

(A) Materials

(B) Methods

a) Isolation of ribosome binding sites (for Section I)

b) Analysis of the ribosome-bound [^{32}P]RNA

c) Preparation of RNAase A-resistant oligonucleotides of Qβ RNA

d) Partial T_1 analysis of the oligonucleotides

e) Wandering spot analysis

(i) Analysis of P-4

(ii) Analysis of P-2a and P-2b

(iii) Analysis of P-15

f) Preparation of Qβ minus strands with N^4-hydroxyCMP substitutions at the positions complementary to the third and fourth nucleodides of the coat cistron

(i) Preparation of the 70 S Qβ RNA-ribosome complex

(ii) Synthesis of minus strands up to the position complementary to the 16th nucleotide of the coat cistron (step 1)

(iii) Stepwise elongation of the minus strand up to the position complementary to the 5th nucleotide of the coat cistron (step 2)

(iv) Incorporation of N^4-hydroxyCMP into the positions complementary to the third and fourth nucleotide of the coat cistron (step 3)

(v) Completion of the minus strand (step 4)

g) Purification and analysis of the minus strands

h) Synthesis of ^{32}P-labeled plus strands using minus strands as template

i) Purification of plus strands

j) Isolation of the ribosome binding site (for Section III)

k) Isolation of the replicase S binding site

l) Two-dimensional polyacrylamide gel electrophoresis

REFERENCES

INTRODUCTION

Initiation of protein synthesis in procaryotes takes place at an A-U-G or, less frequently at a G-U-G triplet (Volckaert & Fiers, 1973; Fiers et al., 1975) which direct the incorporation of formyl-methionine into the amino terminal position of the polypeptide chain (cf. review by Lucas-Lenard & Lipmann, 1971). This step is preceded by the attachment of 70 S ribosome to the initiator region of the mRNA, i.e. the selective binding of ribosomes to form the 70 S initiation complex. The ability of ribosomes to recognize the true protein synthesis initiator codon and ignore many internal or external A-U-G and G-U-G triplet in mRNA has been the subject of intensive study for many years.

It has been proposed by Noll and Noll (1974) and by Jay and Kaempfer (1975) that initiator fMet-tRNA first attaches to the 30 S ribosomal subunit, and that the complex, in a second step, binds to the protein initiation site, with the codon-anticodon playing an essential role in the stabilization of the resulting complex. In view of the obligatory requirement of fMet-tRNA for the formation of the initiation complex reported by these authors, it was somewhat surprising that Vermeer et al. (1973) and Szer and Leffler (1974) were able to detect the efficient formation of complexes of 30 S ribosomes and phage RNA in the absence of fMet-tRNA. Similar observations had been made by Greenshpan and Revel (1969) for T4 mRNA.

A fundamental approach to this problem involved the isolation and nucleotide sequence analysis of the initiator region (or ribosome binding site) of the mRNA, the RNA segment which comprises the initiator triplet, usually in its middle sec-

tion (Steitz, 1975). However, little has been learned about the structural features of the region which distinguish it from other RNA segments containing an A-U-G or G-U-G triplet. On the other hand, it has been known for a long time that the cistron selection specificity resides mainly in the 30 S subunit of the ribosome. For example, (1) E.coli ribosomes initiate synthesis in vitro of all the proteins coded by bacteriophage f2 but the ribosomes from B.stearothermophilus initiate protein synthesis at only one of the cistrons (Lodish, 1970a). (2) Ribosomes from C. crescentus cannot translate any of the MS2 RNA cistrons, while E.coli ribosome cannot translate RNA form C.crescentus phage Cb5 (Leffler & Szer, 1973). It is clearly shown by these reports that the translation specificity depends on the source of the 30 S subunits while the 50 S subunits and initiation factors are interchangeable. Subsequently, the functional importance of several 30 S ribosome components, namely of the ribosomal proteins S 1 (van Duin & van Knippenberg, 1974; van Diejen et al., 1976; Szer & Leffler, 1974; Hermoso & Szer, 1974), and S 12 as well as of the 16 S rRNA (Held et al., 1974; Goldberg & Steitz, 1974) in the initiation of protein synthesis has been documented.

While the real role of those proteins still remains to be clarified, the importance of 16 S rRNA has been further illuminated by Shine and Dalgarno who in 1974 noted that the region preceding the initiation triplet in all mRNAs known at the time always contained a sequence complementary to the 3' terminal region of the 16 S RNA of the 30 S ribosomal subunit. This suggested that hydrogen-bonding between the two sequences served to place the ribosome in the correct position on the mRNA (Shine & Dalgarno, 1974). Their hypothesis was tested by Steitz & Jakes (1975) who showed that an RNA fragment from the R 17 A cistron ribosome binding site, which contained 7

bases complementary to the 3' terminus of 16 S RNA, formed a stable complex with ribosomes, and that the expected RNA-RNA hybrid was released upon cleavage of the complex by colicin E 3. Recently, Dunn et al. (1978) found that a point mutation diminishing the extent of a potential Shine-Dalgarno interaction at the initiator region of 0.3 mRNA of phage T7 causes a shift of ribosome binding to an A-U-G located 15 nucleotides downstream, and a suppressor mutation, which allows a potentially more stable Shine-Dalgarno interaction at the original site restores the original binding capacity of the mRNA. It is assumed that the ribosomal protein S 1 as well as the initiation factor 3, both of which are important for ribosome binding to natural mRNA, facilitate or stabilize such a RNA-RNA interaction (Steitz et al., 1977a). On the other hand, Waltz et al. (1976) reported that λ Prm mRNA, which codes for repressor protein, was completely devoid of a leader sequence and started with the initiator triplet pppA-U-G... . Their work, therefore, suggested that at least in the case of mRNAs lacking a "Shine-Dalgarno" region the A-U-G triplet may play a role in the ribosome binding process. However, it was not clear whether the initiation triplet itself is generally required for the formation of the 70 S initiation complex between ribosomes and mRNAs and if so, to what extent, especially in view of the reports about the existence of the stable 30 S mRNA complex formed in the absence of fMet-tRNA (Vermeer et al., 1973; Szer & Leffler, 1974).

The aim of this paper is to document the importance of two kinds of RNA-RNA interactions in the process of ribosome binding to mRNA, namely that between the 3' terminus of 16 S RNA and the "Shine-Dalgarno region" of mRNA on the one hand, and that of fMet-tRNA with the initiation codon (and possibly its immediate surroundings) on the other. To this purpose,

we have used the RNA of phage Qβ, which serves both as genome and as messenger RNA.

Qβ RNA comprises three cistrons, arranged in the sequence (5')A_2-COAT/A_1-REPLICASE(3') (Weissmann et al., 1973). The middle cistron gives rise mainly to coat protein, and to the longer protein A_1 by the read-through which occurs at the end of the coat cistron (Weissmann et al., 1973). When incubated with Qβ RNA under initiation condition, E.coli ribosomes bind exclusively at the coat cistron initiation site (Hindley & Stamples, 1969).

Three types of experiments were performed: (1) Ribosomes and Qβ RNA were incubated in the presence or in the absence of fMet-tRNA and the ribosome-bound RNA was isolated and characterized. It was found that in the absence of fMet-tRNA ribosomes attach mainly to three regions of the RNA, none of which is part, or in the linear vicinity of a protein initiation site. Moreover, all sequences from these regions show extensive complementarity to the 3' terminal region of 16 S rRNA. (2) The functional importance of the 3' terminus of the 16 S rRNA in the process of 70 S initiation complex formation was examined by blocking the critical region of the 16 S rRNA 3' terminus by a nonanucleotide (P-2a) complementary to it. We show that the oligonucleotide is a potent inhibitor of 70 S initiation complex formation between Qβ RNA and ribosomes, while fMet-tRNA dependent pA-U-G triplet binding to 70 S ribosome is not at all inhibited by the oligonucleotide. (3) To assess the role of the initiation triplet A-U-G for ribosome binding, we generated mutants of Qβ RNA in which this sequence was modified, using the method of site-directed mutagenesis (Flavell et al. 1974; Domingo et al., 1976). The mutant RNA preparation consisted of four Qβ RNA species, with the sequence -A-U-G-G-(wild type), -A-U-A-G-(mutant C_3), -A-U-G-A-(mutant C_4) and -A-U-A-A-

(mutant $C_{3/4}$) at the beginning of the coat cistron. It was found that the relative ribosome binding efficiency of the mutant RNAs to wild type was < 0.1 (mutant C_3), 3.2(mutant C_4) and 0.3(mutant$_{3/4}$).

RESULTS

Section I: Interaction of E.coli ribosomes with Qβ RNA

It has been known for long time that the cistron selection specificity resides in the 30 S subunit of ribosome (Lodish, 1970a; Leffler & Szer, 1973). However, it was not clear whether 30 S ribosome by itself is capable of recognizing the true initiation site in the absence of initiator fMet-tRNA. Several reports argued about the absolute requirement of fMet-tRNA in the process (Noll & Noll, 1974; Jay & Kaempfer, 1975), while others demonstrated the existence of a stable complex formed between 30 S ribosomes and mRNA in the absence of fMet-tRNA (Vermeer et al., 1973; Szer & Leffler, 1974).

In order to clarify this problem and to elucidate further the molecular basis of ribosome-mRNA interactions in the presence or in the absence of fMet-tRNA, we have done the following experiments.

(A) Binding of ribosomes to Qβ RNA in the presence and absence of fMet-tRNA

E.coli 70 S ribosomes (300 pmol) were incubated with [^{32}P]Qβ RNA (160 pmol) under binding conditions, with or without fMet-tRNA. The mixture was digested with RNAase A and centrifuged through a sucrose density gradient. To determine the ribosome-bound RNA each fraction was filtered through a Millipore membrane and the radioactivity retained on a filter was determined. Fig. 1a shows that in the presence of fMet-tRNA 0.23 % of the input radioactivity bound to ribosomes; most of this (0.2 %) sedimented in the 70 S

Fig. 1 Isolation of ribosome-bound Qβ RNA fragments by sucrose centrifugation.

a) ^{32}P-profile of Qβ RNA bound to 70 S and 30 S ribosomes in the presence of fMet-tRNA. Intact ^{32}P-labeled Qβ RNA (about 160 pmol, 6.3 x 10^5 cts/min/pmol) was incubated with ribosomes (300 pmol) under initiation conditions (with added fMet-tRNA). The complex was treated with RNAase A and centrifuged through 5 - 20 % linear sucrose gradient for 2 h at 40,000 rpm and 4 ^{o}C in a SW50 Spinco rotor. Fractions were collected and Millipore-bound radioactivity was monitored throughout the gradient. For details see Materials and Methods.

b) as a) but without addition of fMet-tRNA.

c) ^{32}P-labeled Qβ RNA (about 170 pmol, 3.6 x 10^5 cts/min/pmol) was first completely digested by RNAase A (1 μg RNAase A per 20 μg Qβ RNA, 30 min at 37 ^{o}C, in 120 μl 20 mM Tris-HCl, (pH 7.5), 2 mM EDTA) and then bound to ribosomes (300 pmol) without addition of fMet-tRNA, as in b) (see also text)

●——● , filter-bound radioactivity,

o- - -o , absorbance at 260 nm.

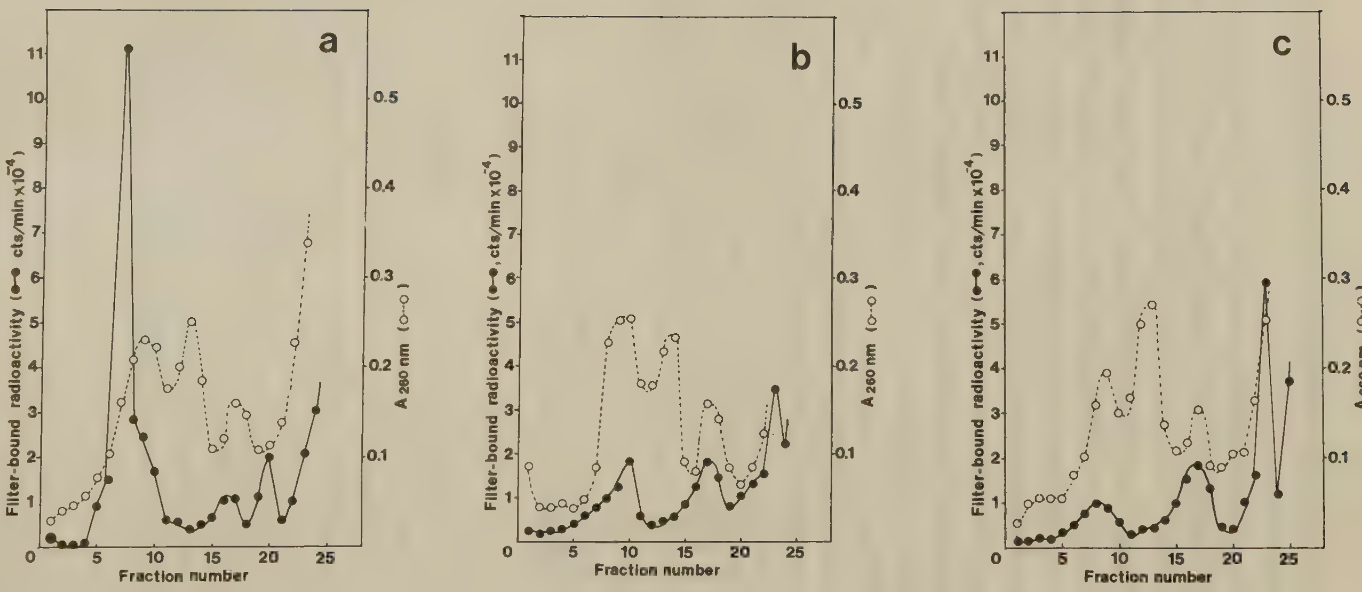

a
b
c
Filter-bound radioactivity (●–●, cts/min x10^-4)
A 260 nm (○--○)
Fraction number
0
5
10
15
20
25
1
2
3
4
5
6
7
8
9
10
11
0.1
0.2
0.3
0.4
0.5

Fig.1

region, and only a small amount (0.03 %) at 30 S. In the absence of fMet-tRNA 0.12 % of the label was bound and distributed equally between the 70 S and the 30 S regions (Fig.1b).

The radioactive RNA was recovered from the pooled 70 S and 30 S regions of both gradients and analyzed by two-dimensional polyacrylamide gel electrophoresis.

The RNA from the 70 S complex formed in the presence of fMet-tRNA yielded 3 major spots, A-6, A-9 and A-10 and about 10 minor spots (cf. Fig. 2a). All oligonucleotides with a chain-length > 10 (designated with the prefix A) were eluted, completely digested with RNAase T_1 and separated by two-dimensional electrophoresis on cellulose acetate and DEAE paper (Sanger & Brownlee, 1967); the T_1 resistant oligonucleotides were further characterized by digestion with pancreatic RNAase A. The products given by A-6, the predominant oligonucleotide, were typical for an RNA fragment derived from the coat cistron ribosome binding site (Fig. 3a, compare to Fig. 4b in Hindley and Staples, 1969). A-9 and A-10 had fingerprints similar to that of A-6, but lacked one or the other T_1-resistant oligonucleotide. The minor oligonucleotides, A-1, A-3, A-4, A-5 and A-8 also represented segments of the coat cistron binding site (data not shown). A-2 (Fig. 3b) and A-7 (data not shown) contained oligonucleotides characteristic for the ribosome binding sites of both the replicase cistron and the coat cistron (Fig. 4). Oligonucleotides with a chain length of 10 or less (designated with the prefix P) were present only at low level; as judged by their position on the gel, three of these appear to be identical with P-2a, P-15 and P-4, as described below.

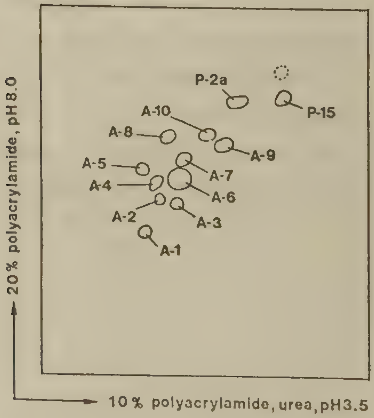
Fig. 2 Electrophoretic analysis of ribosome-bound RNA isolated under various conditions.

The ^{32}-P-labeled RNA fragments bound to 70 S and 30 S ribosomes in the experiment of Fig. 1 were eluted from the Millipore filter, precipitated by ethanol and dissolved in 0.5 mM EDTA. After denaturation, the RNA was analyzed by two-dimensional polyacrylamide gel electrophoresis (De Wachter & Fiers, 1972), and an autoradiogram was prepared (the details are described in the Materials and Methods section).

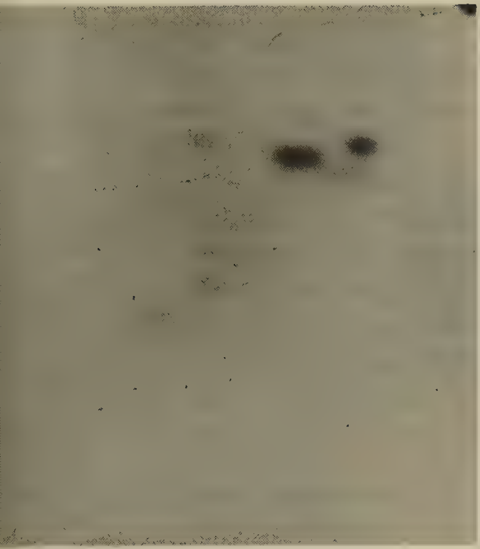
a) 70 S ribosome-bound RNA and
b) 30 S ribosome-bound RNA obtained by incubatin intact RNA with ribosomes in the presence of added fMet-tRNA (cf. Fig. 1a and b, respectively).
c) 70 S ribosome-bound RNA and
d) 30 S ribosome-bound RNA obtained by incubating intact RNA with ribosomes without additon of fMet-tRNA (cf. Fig. 1b), a') to d'), tracings of a) to d), respectively.

a

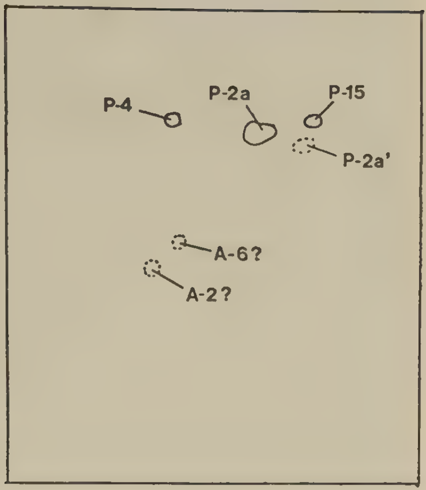
a'



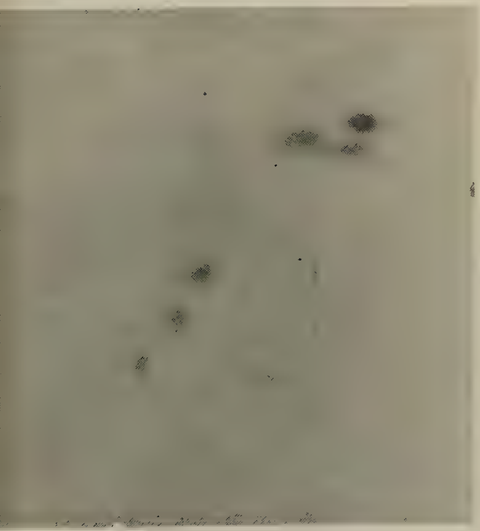
b



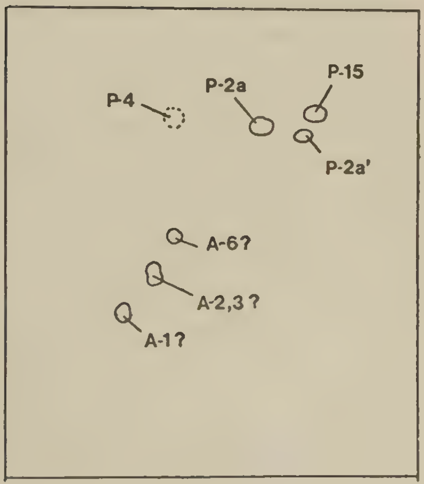
b'



c



c'



d

d'

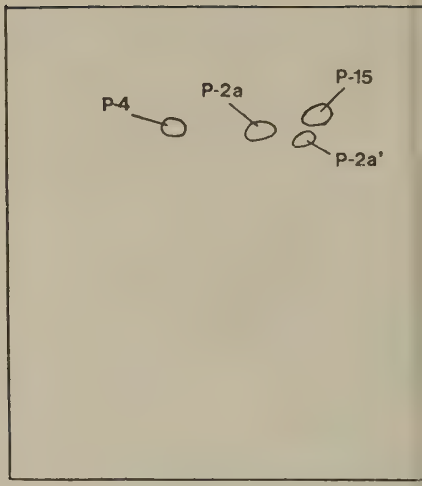
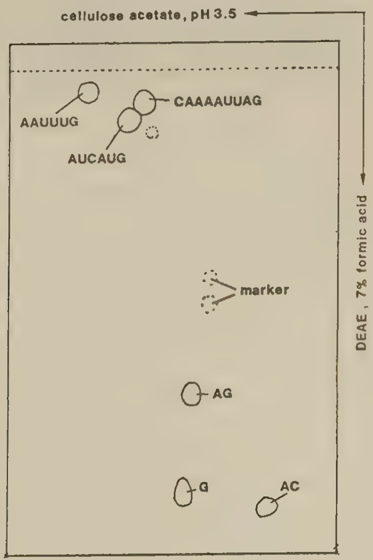


Fig. 3 T_1-fingerprint analysis of large ribosome-bound [^{32}P] oligonucleotides.
The isolation and purification of ribosome-bound RNA was described in the legends to Fig. 1 and 2. The RNA was eluted from the gel, digested with RNAase T_1, analyzed by two-dimensional electrophoresis on cellulose acetate and DEAE cellulose, and autoradiographed as described by Taniguchi and Weissmann (1978).
a) Fragment A-6, b) fragment A-7, a') and b') tracings of a) and b), respectively. The T_1 oligonucleotides were identified by their mobility and their RNAase A digestion products by comparison with the data of Steitz (1972) and Taniguchi and Weissmann (1978).

a

a'



b

b′

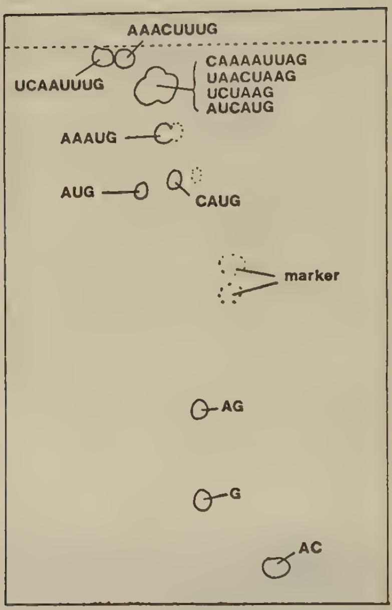
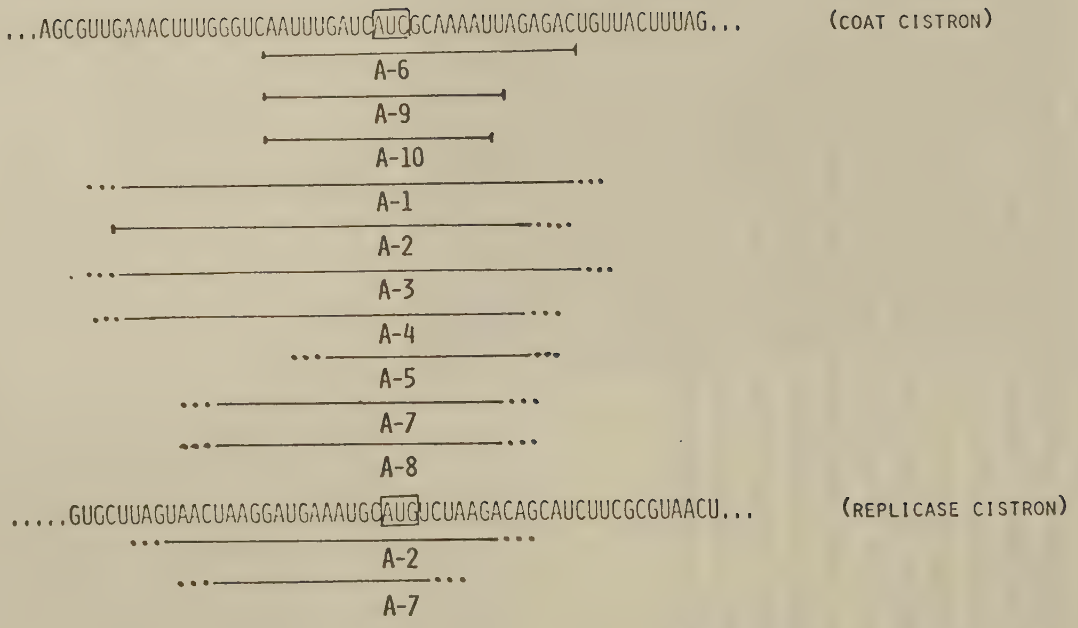

AAACUUUG
UCAAUUUG
CAAAAUUAG
UAACUAAG
UCUAAG
AUCAUG
AAAUG
AUG
CAUG
marker
AG
G
AC

Fig. 4 Origin of large [^{32}P]oligonucleotides recovered from the 70 S ribosome-Qβ[^{32}P]RNA complex.
The large RNA fragments (A-1 to A-10) were recovered after RNAase A digestion of the 70 S ribosome-Qβ [^{32}P]RNA complex formed in the presence of fMet-tRNA and subjected to T_1 fingerprint analysis as described in the legend to Fig. 3. The T_1 oligonucleotides were identified by their mobility as well as by their RNAase A digestion products, by comparison with the data of Steitz (1972) and Taniguchi and Weissmann (1978). The origin of the fragments was deduced by fitting the oligonucleotides to the known sequence around the ribosome binding site (Weissmann et al., 1973). Dotted lines: assignment uncertain.



...AGCGUUGAAACUUUGGGUCAAUUUGAUCAUGGCAAAAUUAGAGACUGUUACUUUAG...
(COAT CISTRON)
A-6
A-9
A-10
A-1
A-2
A-3
A-4
A-5
A-7
A-8
.....GUGCUUAGUAACUAAGGAUGAAAUGCAUGUCUAAGACAGCAUCUUCGCGUAACU...
(REPLICASE CISTRON)
A-2
A-7

Fig.4

Two-dimensional polyacrylamide gel electrophoresis of the [^{32}P]RNA from the 30 S region (see Fig. 1b) revealed two major products, P-15 and P-2a, and two minor products, P-2a' and P-4, both of low molecular weight as judged by their mobilities (Fig. 2b). All products were resistant to further digestion by RNAase A except for P-2a', which on complete digestion yielded Up and P-2a. As will be shown below, P-2a, P-4 and P-15 are not derived from any of the three initiation regions of Qβ RNA, nor from their vicinity. Only traces of oligonucleotides with the mobility of A-2 (initiator regions of coat and replicase cistron) and A-6 (coat initiator region) were detected.

When the [^{32}P]RNA from the 70 S complex formed in the absence of (added) fMet-tRNA was subjected to the same analysis, only about 30 % of the counts were found in oligonucleotides from the coat cistron initiation site, the rest of the radioactive material was located in the position of P-4, P-15 and P-2a (Fig. 2c). The 30 S ribosome-bound RNA consisted of only the small RNAase A-resistant oligonucleotides P-4, P-15, P-2a and P-2a' (Fig. 2d). The oligonucleotides were characterized by complete digestion with RNAase T_1 (see below).

Assuming that the average chain length of the RNA from the initiation site is 25 (Hindley & Staples, 1969) and that from the non-initiator regions is 9 (see below) the initiator site RNA bound to 70 S ribosomes, in the presence of fMet-tRNA, was about 77 mol % of the defined radioactive oligonucleotides detectable on the autoradiogram. Without addition of fMet-tRNA the value was 15 %. The RNA fragments from non-initiator regions (P-4, P-15, P-2a and P-2a') bound to 70 S ribosome was 23 mol % in the presence of fMet-tRNA and 85 mol % without the addition of fMet-tRNA. The 30 S region

yielded only traces of oligonucleotides derived from an initiation site in the presence of added fMet-tRNA and none at all in its absence.

Assuming that all ribosome-bound oligonucleotides are recovered with similar efficiency (on average 55 %; Billeter et al., 1974), and taking into account the recovery from the filter (70 %) we calculate that 16.0 pmol and 0.8 pmol of initiator region RNA were bound to 300 pmol 70 S ribosomes in the presence or absence of added fMet-tRNA, respectively. This represents 10.0 mol % and 0,5 mol % of the input [^{32}P]RNA (160 pmoles). For comparison it may be noted that when Qβ[^{32}P]RNA is bound to ribosomes in the presence of fMet-tRNA and centrifuged through a sucrose gradient without prior RNAase A digestion about 18 % of the radioactivity is recovered in the 70 S region. The amount of RNA from non-initiator regions (P-4, P-15, P-2a) bound to 70 S ribosome was 4.9 pmol and 4.8 pmol in the presence and in the absence of fMet-tRNA, respectively. When the 30 S region was examined, the values were 3.3 pmol and 9.8 pmol, respectively. The analysis is summarized in Table 1.

In a further experiment Qβ[^{32}P]RNA (170 pmol) was first completely degraded with RNAase A and then bound to unfractionated ribosomes (300 pmol) without addition of fMet-tRNA. Radioactivity was detected both in the 70 S and the 30 S region (Fig. 1c). The analysis of the 30 S and 70 S ribosome-bound [^{32}P]RNA revealed that mainly oligonucleotide P-2a was present, in yields of 65 and 83 mol %, respectively (Table 1). It thus appears that the ribosomes bind preferentially to the P-2a sequence when it is accessible; this is not entirely the case in intact RNA, where ribosomes bind to P-15 and P-4 as well as to P-2a. Why are P-15 and P-4 not bound to ribosomes from an RNAase A digest of Qβ RNA? It is possible that, although a molar excess of ribosomes was used, not all ribo-

TABLE 1 Analysis of $[^{32}P]$RNA fragments obtained from ribosome Q RNA complexes formed under various conditions

Expt.*	a) Conditions for complex formation	b) Peak (S)	c) Radioactivity retained on Millipore (cpm (% of input))	d) Total radioactivity recovered in defined oligonucleotides (cpm (% of input))	e) Distribution of radioactivity among defined oligonucleotides (% of total, (pmol of RNA))+: coat initiation site	replicase initiation site	P-15	P-2a + P-2a'	P-4	unknown
	fMet-tRNA present, Qβ$[^{32}P]$ RNA intact (1×10^8 cpm)	70	2×10^5 cpm (0.20)	2.5×10^4 cpm (12.5)	80.4 (14.8)	6.3 (1.2)	2.3 (1.3)	6.9 (3.6)	0	4.1
		30	3.2×10^4 cmp (0.03)	1.8×10^3 cpm (5.5)	barely detectable	0	32.9 (1.4)	57.0 (1.5)	10.0 (0.4)	0
2	no fMet-tRNA, Qβ$[^{32}P]$RNA intact (1×10^8 cpm)	70	6×10^4 cpm (0.06)	3.3×10^3 cpm (5.5)	33.7 (0.8)	0	28.0 (2.2)	31.3 (2.1)	6.9 (0.5)	0
		30	6×10^4 cpm (0.06)	4.5×10^3 cpm (7.5)	0	0	34.8 (3.7)	53.5 (5.0)	11.7 (1.1)	0
3	no fMet-tRNA, Qβ$[^{32}P]$RNA predigested (6.2×10^7 cpm)	70	3.4×10^4 cpm (0.05)	6.2×10^3 cpm (11.0)	0	0	9.3 (1.3)	83.2 (10.4)	0	7.5
		30	6.2×10^4 cpm (0.1)	6.2×10^3 cpm (10.0)	0	0	6.7 (1.6)	65.0 (13.5)	0	28.3

TABLE 1 (cont.)

+ Corrected value = measured value x $\frac{1}{0.7}$ X $\frac{1}{0.55}$ where 0.7 is the recovery of radioactivity from the filter (c) and 0.55 the recovery of a defined oligonucleotide after two-dimensional polyacrylamide gel electrophoresis (Billeter et al., 1974). The overall recovery of the filter-bound radioactivity (c) in defined spots of the two-dimensional gel was 5 - 15 %. Typically, 70 % of the filter-bound radioactivity could be extracted and applied to the gel. In one experiment we determined that 24 % of the radioactivity were present in defined spots (e) while 58 % were distributed over the remainder of the gel. The diffusely distributed material may be partly due to random oligonucleotides unspecifically bound to the ribosomes and partly to streaking.

* In experiments 1 and 2, 160 pmol of Qβ[^{32}P] RNA (specific radioactivity, 6.3 x 10^5 cts/min/pmol), 300 pmol ribosome and where indicated 450 pmol fMet-tRNA were incubated under binding conditions, treated with RNAase A and centrifuged through a sucrose gradient as detailed in the Materials and Methods section. In experiment 3, 170 pmol Qβ[^{32}P]RNA (specific activity 3.6 x 10^5 cts/min/pmol) were first completely digested by RNAase A and then bound to 300 pmol of ribosomes without addition of fMet-tRNA. The sedimentation patterns are shown in Fig. 1. The radioactive RNA in the 70 and 30 S peaks was extracted and analyzed as shown in Figs. 2 and 3.

somes were active in binding. Moreover, P-2a, which has a strong binding affinity, and a large variety of other oligonucleotides with lower affinity may have competed with the binding of P-15 and P-4. The question arose as to whether, in binding experiments with intact Qβ RNA, attachment of ribosomes to P-2a occurred only after the RNA had been cleaved by RNAase A. Ribosomes were bound to Qβ[^{32}P] RNA in the absence of fMet-tRNA, and RNAase A was added in the presence or absence of unlabeled RNAase A-digested Qβ RNA, in 4-fold molar excess over ribosomes and 8-fold molar excess over Qβ[^{32}P]RNA. As shown in Table 2 about 0.03 % of the input ^{32}P -radioactivity were recovered in the 30 S region whereas 0.05 % of the radioactivity were found without addition of unlabeled Qβ RNA. An analysis by two-dimensional polyacrylamide gel electrophoresis of the fragment bound to 30 S ribosomes revealed that in the presence of excess unlabeled Qβ RNA ^{32}P-labeled P-15 was diminished by 71.5 %, but ^{32}P-labeled P-2a by only 24 %. Under similar conditions but without RNAase treatment, no significant breakdown of Qβ RNA was observed during the incubation of the RNA with ribosomes (data not shown; see Taniguchi & Weissmann, 1978). The experiments show that 30 S ribosome interaction with P-2a occurred with intact Qβ RNA, since otherwise a 4- to 6-fold reduction of ribosome-bound [^{32}P]P-2a should have resulted. The reduction of ^{32}P-labeled P-15 may be due to an exchange with P-2a and/or other Qβ RNA fragments present in excess.

In summary, the results of this sections show that in the absence of fMet-tRNA ribosomes bind almost exclusively to non-initiator regions of Qβ RNA and in its presence predominantly to the coat cistron initiator region.

TABLE 2 Recovery of non-initiator [^{32}P]oligonucleotides from 30 S ribosome Qβ[^{32}P]RNA complexes digested with RNase A in the presence or absence of excess unlabeled Qβ RNA

a) Unlabeled Qβ RNA	b) Radioactivity retained on filter (cpm (% of input))	c) Radioactivity recovered in defined spots on gel (cpm (% of b))	Distribution of radioactivity (% of total (pmol of RNA))		
			P-15	P-2a	unknown
none	4.2×10^4 (0.05)	6.7×10^3 (13.9)	22.0 (0.7)	73.6 (2.1)	4.4
8-fold excess over Qβ[^{32}P] RNA	2.8×10^4 (0.03)	4.4×10^3 (15.7)	8.6 (0.2)	83.7 (1.6)	7.7

TABLE 2 (cont.)

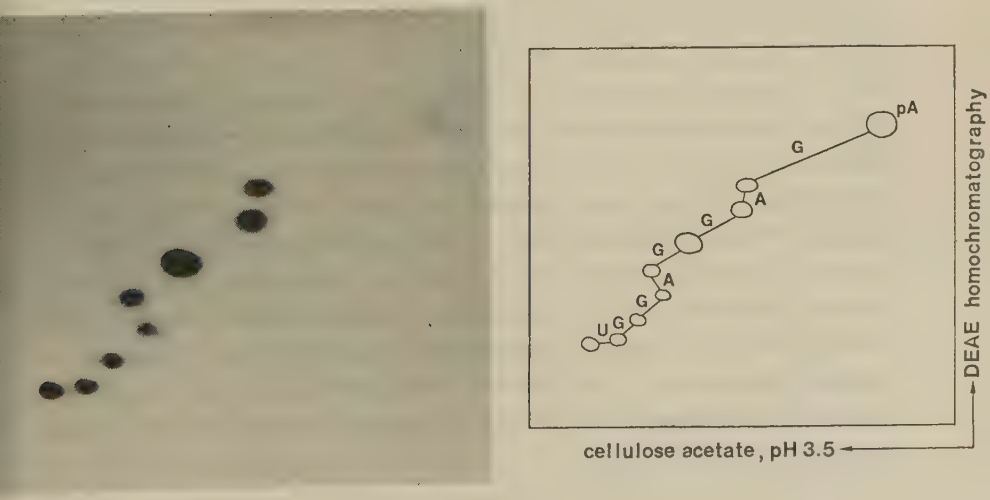
Two reaction mixtures, each containing Qβ[^{32}P] RNA (33 pmol, specific activity 2.6 x 10^6 cts/min/pmol) and ribosomes (75 pmol) were incubated under ribosome binding conditions, in a final volume of 50 µl as described in the Materials and Methods section, however without addition of fMet-tRNA. To one sample about 270 pmol of unlabeled Qβ RNA were added; both samples were digested with RNAase A (0.5 µg in the tube without and 2 µg in the tube with additional unlabeled RNA) and the ribosome-bound [^{32}P]RNA was analyzed by two-dimensional polyacrylamide gel electrophoresis as in the experiment of Fig. 2.

(B) The structure of oligonucleotides P-2a, P-2b, P-4 and P-15

Qβ [^{32}P]RNA was completely digested with RNAase A, the products were separated by two-dimensional polyacrylamide gel electrophoresis and the spots designated P-2, P-4 and P-15 were eluted. An aliquot of P-4, digested to completion with RNAase T_1, yielded 3 A-Gp, 2 Gp and Cp, the same products obtained on digestion of oligonucleotide P-4 isolated from a ribosome binding experiment. P-2 was present in about twice the molar yield of P-4 and yielded 0.5 A-A-Gp, 2 A-Gp, 2.5 Gp, 1.00 Up, showing that at least 2 oligonucleotides were present. Two-dimensional homochromatography (Volckaert et al., 1976) partially resolved two oligonucleotides, P-2a and P-2b. On digestion with RNAase T_1, P-2a yielded 3 A-Gp, 2 Gp, Up and P-2b A-A-Gp, A-Gp, 3 Gp, Up. Separation in this system was marginal and inadequate for the purification of larger quantities of oligonucleotide. Therefore, the oligonucleotide mixture P-2 was incubated with an excess of ribosomes and centrifuged through a sucrose gradient. The ribosome-bound and the non bound [^{32}P]RNA were characterized as P-2a and P-2b, respectively, by digestion with RNAase T_1 and analysis of the products.

The nucleotide sequences of oligonucleotides P-2a and P-2b were determined by 5' terminal labeling with ^{32}P phosphate using polynucleotide kinase, partially digesting the products with snake venom exonuclease and separating the products by electrophoresis and homochromatography ("wandering spot" method (Rensing & Schoenmakers, 1973). As documented by Fig. 5, the sequence of P-2a was pA-G-A-G-G-A-G-G-Up.

Fig. 5 Nucleotide sequence analysis of P-2a.
A mixture of oligonucleotides P-2a and P-2b was obtained by complete RNAase A digestion of Qβ[^{32}P] RNA (about 400 pmol, specific activity 8.4 x 10^4 cts/min/pmol) and two-dimensional polyacrylamide gel electrophoresis (De Wachter & Fiers, 1972). The RNA was eluted from the appropriate region of the gel and labeled at the 5' end with polynucleotide kinase and [γ-^{32}P]ATP (specific activity 1.1 mCi/nmol). P-2a was purified from P-2b by ribosome binding as described in Materials and Methods. The 5'-^{32}P-labeled RNA (9.5 x 10^4 cts/min) was partially digested by snake venom phosphodiesterase and analyzed essentially as described by Rensing and Schoenmakers (1973).



Whereas P-2a was isolated in pure form, P-2b was present in a mixture with P-2a. Analysis of this mixture gave two tracks, one of which was due to P-2a; the other gave the sequence pG-G-G-A-G-A-A-G-Up.

Uniformly [^{32}P]-labeled P-15, recovered from ribosome binding experiments, was digested with RNAase T_1 and the products were characterized. The relative values for AG, G and U (average of 3 independent experiments) were 1.2 ($\pm$ 0.25) : 3.9 ($\pm$ 1): 1. For further analysis, ^{32}P-labeled Qβ RNA was totally digested with RNAase A and the products were separated by two-dimensional polyacrylamide gel electrophoresis. The oligonucleotide with the mobility of P-15 was isolated, labeled at the 5' terminus with ^{32}P at high specific radioactivity and subjected to "wandering spot" analysis (data not shown). The major track, pA-G-G-G-G-G-U was compatible with the T_1-analysis of P-15 recovered from ribosomes; a minor track, pG-G-G-G-G-G-C was detected as well and must be due to a minor component which was not bound to ribosomes under the conditions of our experiment.

For the sequence determination of P-4, uniformly ^{32}P-labeled P-4 was partially digested by spleen exonuclease and the products were separated in two dimensions as described above; the sequence was G-G-A-A-G-G-A-G-Cp (data not shown). The nucleotide sequences of P-2a, P-2b and P-4 were further confirmed by partial RNAase T_1 digestion followed by two-dimensional electrophoresis (Sanger & Brownlee, 1967) (results not shown). None of the oligonucleotides described are located in the vicinity of a Qβ protein initiation site, as shown by inspection of the known Qβ RNA sequences (Weissmann et al., 1973).

(C) Interaction of oligonucleotide P-2a with the 3'end of 16 S rRNA within the 30 S ribosome

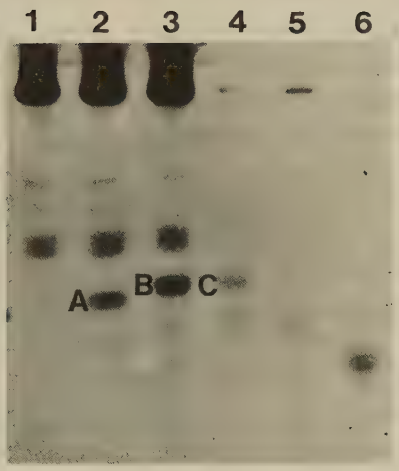
The fact that the nonanucleotide P-2a has a sequence of eight bases complementary to the 3' end of 16 S rRNA strongly suggested that the interaction of the 30 S ribosome with the Qß RNA sequence corresponding to P-2a (and possibly to P-15 and P-4) is mediated by an RNA-RNA interaction similar to the one proposed by Shine and Dalgarno (1975) (Fig. 7). In order to examine this question, an experiment similar to that of Steitz and Jakes (1975) was carried out. A mixture of about 40 % P-2a and 60 % P-2b (both ^{32}P-labeled) was used in these experiments, since the two oligonucleotides are difficult to separate. It was shown above that only P-2a was bound to ribosomes. The oligonucleotides were incubated with ^{32}P-labeled and unlabeled E.coli ribosomes, respectively, and an aliquot of each preparation was digested with cloacin DF13, a bacteriocin similar to colicin E3, which specifically cleaves 16 S rRNA within the interact ribosome, releasing a 3' terminal fragment of 49 nucleotides (de Graaf et al., 1973). The samples, as well as appropriate controls, were treated with 1 % sodium dodecyl sulfate and analyzed by polyacrylamide gel electrophoresis (Fig. 6a). Lane 1 shows that in the case of untreated [^{32}P]ribosomes most of the radioactivity did not enter the gel. and only minor bands corresponding to 5 S RNA and tRNAs appeared, as reported earlier by Steitz and Jakes (1975). [^{32}P]ribosomes incubated with cloacin DF13 yielded a radioactive band at about the position corresponding to a 50-mer (band A in lane 2). ^{32}P-labeled ribosomes which were incubated with ^{32}P-labeled oligonucleotide P-2 (a+b) and then treated with cloacin DF13 showed an intense band slower than band A (band B in lane 3). Unlabeled ribosomes which had been first incubated with ^{32}P-labeled oligonucleo-

Fig. 6 Analysis of the products resulting form cloacin DF13 cleavage of the complex formed by incubation of ribosomes and oligonucleotides P-2 (a+b).

a) Six pmol of ^{32}P-labeled (specific activity, 8.3 x 10^4 cts/min/pmol) or unlabeled ribosomes were incubated with 16 pmol of ^{32}P-labeled P-2(a+b)(a 2:3 mixture of P-2a and P-2b; specific activity 1170 cts/min/pmol) in 10 µl of 60 mM Tris-HCl (pH 7.5), 120 mM NH_4Cl, 8 mM $MgCl_2$, 0.6 mM GTP, 0.6 mM dithiothreitol at 37 °C. After 10 min, 0.8 µg of cloacin DF13 were added and the mixture was further incubated for 20 min at 37 °C. The complex was dissociated 15 min at 0 °C in 1% sodium dodecyl sulfate, then loaded on a 9% polyacrylamide slab gel (20 x 40 x 0.2 cm), in 40 mM Tris-acetate (pH 7.4), 5 mM $MgCl_2$. After electrophoresis at 4 °C and 200 V for about 15 h an autoradiogram was prepared. Slot 1: ^{32}P-labeled ribosomes without cloacin digestion. Slot 2: ^{32}P-labeled ribosomes digested with cloacin. Slot 3: ^{32}P-labeled ribosomes incubated with ^{32}P-labeled P-2(a+b) and digested with cloacin. Slot 4: unlabeled ribosomes incubated with ^{32}P-labeled P-2(a+b) and then digested with cloacin. Slot 5: unlabeled ribosomes incubated with ^{32}P-labeled P-2(a+b) alone.

b) RNA from bands A, B and C was extracted from the gel of Fig. 6a, precipitated by ethanol and dissolved in 10 µl 0.5 mM EDTA, 4,5 mg of urea were added and the solution heated for 90 sec at 70 °C. The samples were analyzed on a 15% polyacrylamide vertical slab gel (20 x 40 x 0.2 cm) in 0.1 M Tris-borate (pH 8.3), 50 mM EDTA, 7 M urea. Electrophoresis was for about 15 h at room temperature, at 400 V.

a



b

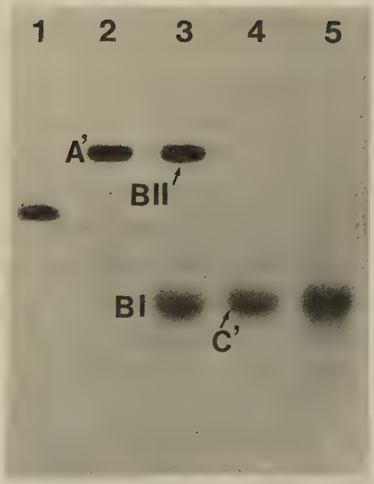


Fig.6

tide P-2 (a+b) and then treated with cloacin DF13 yielded a radioactive band with the same mobility as band B, as well as a minor band remaining on the top of the gel (band C in lane 4). When the cloacin tretment was omitted, most radioactivity remained at the top of the gel (lane 5).

No free oligonucleotide P-2b was detected, although, as shown below, only P-2a is bound to ribosomes. This is most likely due to nucleases present in the ribosome preparation, which rapidly degrade free, but not bound oligonucleotides. Slot 6 shows the ^{32}P-labeled oligonucleotide P-2 (a+b) alone as a diffuse band in the position characteristic for a decanucleotide. The labeled RNA released by cloacin treatment (bands A, B and C) was eluted from the gel, denatured and analyzed by polyacrylamide gel electrophoresis in 7M urea (Fig. 6b). The [^{32}P]RNA form band A (released from ^{32}P-labeled, uncomplexed ribosomes) gave a single band, A' (lane 2) which, by T_1 fingerprint analysis (data not shown) was identical with the 3' terminal region of 16 S RNA (compare with de Graaf et al., 1973). The [^{32}P]RNA from band B (derived from the complex formed by incubating ^{32}P-labeled P-2(a+b) with ^{32}P-labeled ribosomes) dissociated into two products, BI and BII (lane 3). RNAase T_1 digestion of the [^{32}P]RNA eluted from BI yielded 3.2 A-Gp, 2.2 Gp and 1.0 Up, the products characteristic for P-2a, and almost no A-A-Gp (0.05), which would have indicated the presence of P-2b. Thus, from a mixture of P-2a and P-2b, essentially only P-2a was bound to ribosomes. BII had the mobility and the T_1 digestion products characteristic for the 3' terminal region of 16 S RNA. The labeled product from band C (released from the complex formed by incubating ^{32}P-labeled P-2(a+b) with unlabeled ribosomes) gave a single band C' (lane 4) with the mobility and the T_1 digestion products typical for P-2a.

Fig. 7 Base complementarities between ribosome binding sites of Qβ RNA and the 3' terminal region of E.coli 16 S rRNA.

* The nucleotide sequence of initiator regions and the 3' terminal nucleotide sequence of E.coli 16 S rRNA were taken from Steitz (1975) and Shine and Dalgarno (1975). The sequence of the B.stearothermophilus sequence is from Sprague et al. (1977).

† ΔG was calculated according to Gralla and Crothers (1973) for the optimal interaction with the 3' terminal segment of E.coli 16 S rRNA, without considering the free energy contribution resulting from the bimolecular nature of the reaction.

	Sequence	ΔG (kcal/mol)† E. coli	ΔG (kcal/mol)† B. stearothermophilus
A) Initiator regions			
Coat cistron	...A-C-U-U-U-G-G-G-U-C-A-A-U-U-U-G-A-U-C-A-U-G-G-C-A-A-A-A... (3') [fMet: A-U-G; Ala: G-C-A; Lys: A-A-A]	-7.9	-7.9
Replicase cistron	...G-U-A-A-C-U-A-A-G-G-A-U-G-A-A-A-U-G-C-A-U-G-U-C-U-A-A-G... (3') [fMet: A-U-G; Ser: U-C-U; Lys: A-A-G]	-10.1	-9.0
A_2 cistron	...A-C-U-G-A-G-U-A-U-A-A-G-A-G-G-A-C-A-U-A-U-G-C-C-U-A-A-A... (3') [fMet: A-U-G; Pro: C-C-U; Lys: A-A-A]	-7.9	-8.5
B) Non-initiator regions			
P-2A	...A-G-A-G-G-A-G-G-U... (3')	-16.9	-16.4
P-15	...A-G-G-G-G-G-U...... (3')	-14.2	-14.2
P-4	...G-G-A-A-G-G-A-G-C... (3')	-11.1	-12.7
C) Non-initiator regions bound by B. stearothermophilus ribosomes*			
Non-initiator A	...C-U-G-A-A-A-G-G-G-G-A-G-A-U-U-A-C-U-C-G...	-9.5	-12.7
Non-initiator B	identical to P-4		

(3') $_{HO}$A-U-U-C-C-U-C-C-A-C-U-A-G... 3' end of 16 S rRNA from E. coli

(3') $_{HO}$A-U-C-U-U-U-C-C-U-C-C-A-C... 3' end of 16 S rRNA from B. stearothermophilus

Fig.7

The experiments described in this section thus show that oligonucleotide P-2a binds to the 3' terminal region of the 16 S rRNA, and that cloacin cleaves off a complex consisting of P-2a and the 3' terminus of the ribosomal RNA.

Section II: Inhibition of Qβ RNA 70 S ribosome initiation complex formation by an ologonucleotide complementary to the 3' terminal region of E.coli 16 S ribosomal RNA

The hypothesis of Shine & Dalgarno predicts that if the 3' terminal region of the 16 S rRNA were blocked, binding of the ribosomes to the initiator region of a mRNA should be diminished or prevented, while binding of the trinucleotide A-U-G should not be affected.

It has been shown in previous section that the oligonucleotide P-2a which is complementary to a sequence of 8 bases at the 3' terminal region of 16 S rRNA (ΔG = -16.9 Kcal/mol) binds tightly to the 30 S ribosome and is released as a complex with the 3' terminal 49-mer of 16 S rRNA after digestion with cloacin DF13. Fig. 8 shows the effect of preincubating ribosomes (5 pmol) with different amounts of purified P-2a prior to the addition of ^{3}H-labeled Qβ RNA (2 pmol). It is seen that the amount of [^{3}H]Qβ RNA in the 70 S peak is about 0,4 pmol (20 % of the input) in the absence of P-2a (Fig. 8a) and decreases with increasing amounts of P-2a. At a molar ratio of P-2a to Qβ RNA of 2.5, 50 % inhibition of 70 S complex formation was observed, and at a ratio 10, inhibition was 87 % (Fig. 8b, Table 3). The effect of several other oligonucleotides on 70 S complex formation was compared to that of P-2a and the results are shown in Table 3.

Fig. 8 Effect of oligonucleotide P-2a on bining of Qβ [^{3}H]RNA to 70 S ribosomes.

The preparation of the pancreatic oligonucleotide P-2a has been described (Taniguchi and Weissmann, submitted for publication). Qβ[^{3}H]RNA, fMet-tRNA and ribosomes were prepared as described (Kolakofsky et al., 1973). Low salt-washed ribosomes (5 pmol) were incubated with or without P-2a (20pmol, specific activity 130 cpm/pmol) in 50 mM Tris-HCl (pH 7.5), 6 mM $MgCl_2$, 120 mM NH_4Cl, 0.5 mM GTP, 0.5 mM dithiothreitol and about 440 pmol of unfractionated, stripped tRNA charged with fMet (estimated to contain 10 pmol of fMet-tRNA) in a volume of 10 µl for 15 min at 37 °C. Then, 2 pmol of Qβ[^{3}H]RNA (specific activity, 3 x 10^4 cpm/pmol; in 2 µl) were added to the reaction mixture and incubation was continued for 10 min at 37 °C. Hundred µl of buffer B (10mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 50 mM NH_4Cl and 10 mM β-mercaptoethanol) were added and the sample was loaded on 4 ml 10 to 30% sucrose gradient in buffer B. Centrifugation was for 90 min at 58,000 rpm and 4 °C in an SW60 Spinco rotor. Twentyone fractions were collected on paper discs (Schleicher & Schuell, LS78 Rufi, Ø 22 mm) and ^{3}H radioactivity was determined in POPOP-scintillator. The OD_{260} profile of a mixture of 30 S, 50 S and 70 S ribosomes, run under the same conditions, was used to locate the ^{3}H-radioactivity sedimenting at 70 S (indicated by (a) in the figure). The amount of 70 S ^{3}H-radioactivity was calculated relative to the total ^{3}H radioactivity recovered from the gradient (a+b). (I) without P-2a; (II) with P-2a (20 pmol).

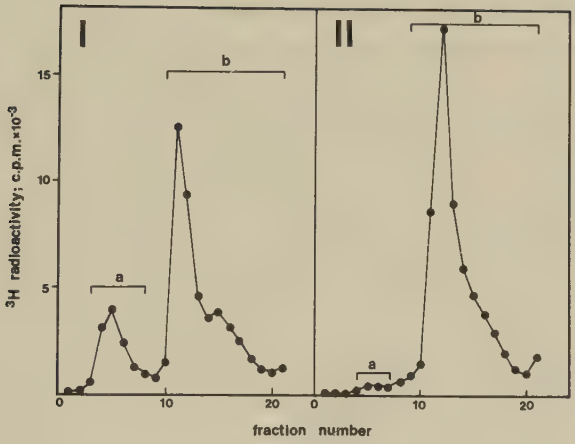

I
II
a
b
15
10
5
0
10
20
3H radioactivity; c.p.m.×10-3
fraction number

Fig.8

An equmolar mixture of P-2b, which has only 5 base complementary towards 16 S rRNA 3' end (see previous section), and P-2a, had the same inhibitory effect as pure P-2a. To show that inhibitor other than an oligonucleotide, an aliquot of the mixture of (weakly ^{32}P-labeled) P-2a and P-2b was treated with Sepharose-linked RNAase T_1; the equivalent of 20 pmol of the ^{32}P-labeled oligonucleotides, which after this treatment was completely digested (as shown by electrophoretic analysis) gave virtually no inhibition (Table 3). The RNAase A-resistant oligonucleotides G-G-A-A-G-G-A-G-C_{OH}(P-4) and G-G-A-G-A-A-G-C_{OH}(P-6), which showed less complementarity with the 3' terminal region of 16 S rRNA (Table 3), were far weaker inhibitors than P-2a; the concentrations required to give 50 % inhibition were about 3 and 5 times greater, respectively, than those required with P-2a. Two pyrimidine-rich RNAase T_1-resistant oligonucleotides, T-14 and T-22, gave virtually no inhibition at a molar ratio of 10 relative to Qβ RNA (Table 3). It is interesting to note in this connection that AG-rich random copolymers were found to be potent competitors against natural mRNA binding to ribosomes (Revel & Greenshpan, 1970).

In a further experiment, a mixture of 5' ^{32}P-labeled pA-U-G (100 pmol) and Qβ [^{3}H]-RNA (2 pmol) was incubated with ribosomes (10 pmol) and 70 S complex formation monitored by sucrose density gradient centrifugation. As shown in Fig. 9, the binding of Qβ RNA as well as A-U-G triplet was strongly dependent on the addition of fMet-tRNA to the incubation mixture: 0.3 pmol of Qβ RNA (15 % of input) and 0.6 pmol of A-U-G (0.6 % of input) were bound in either case when the tRNA was omitted. The binding of A-U-G was almost proportional to its input level, up to 100 pmol.

TABLE 3 Inhibition of binding of 70 S ribosomes to Qβ RNA by oligonucleotides *

Oligonucleotide	Nucleotide sequence	(ΔGKcal./mol)	Amount added (pmol oligo-nucleotide)	% of Qβ[^{3}H]RNA bound to 70 S ribosomes (relative to control$^+$)	% of inhibition
None			none	100	0
P-2a (purified by ribosome binding)++	A-G-A-G-G-A-G-G-U_{OH}	(-16.9)	5	42.6	57.4
" "			10	32.6	67.4
" "			20	12.8	87.2
P-2(a+b) (1:1 mixture)	A-G-A-G-G-A-G-G-U_{OH} (a) G-G-G-A-G-A-A-G-U_{OH} (b)	(-16.9) (-10.0)	20	8.8	91.2
P-2(a+b), treated with RNAase T_1 †			20	115.0	0
P-4	G-G-A-A-G-G-A-G-C_{OH}	(-11.1)	20	43.4	56.6
P-6	G-G-A-G-A-A-G-C_{OH}	(-7.9)	20	57.5	42.6
T-14	A-U-U-U-C-U-U-C-U-A-U-G_{OH}	(-1.1)	20	105.0	0
T-22	A-U-A-U-U-A-U-U-U-U-A-C-C-G_{OH}	(-1.1)	20	90.0	10.0

TABLE 3 (cont.)

*Low-salt washed ribosomes (5 pmol) were incubated with oligonucleotides under the conditions described in the legend to Fig. 1, for 15 min at 37 °C, then Qβ[^{3}H] RNA (2 pmol, specific activity 6.9 x 10^4 cpm/pmol or 3 x 10^4 cpm/pmol) was added to the mixture and incubation continued for 10 min at 37 °C. The mixture was diluted with 100 μl of buffer B and analyzed by 10-30% sucrose gradient centrifugation as described (Taniguchi & Weissmann, 1978).

+In the control experiment where oligonucleotide was omitted, about 20 % of the input Qβ[^{3}H]RNA was bound to 70 S ribosomes (cf. Fig. 1a).

++Taniguchi and Weissmann, submitted for publication.

†BrCN-activated Sepharose 4B was prepared and RNAase T_1 was immobilized essentially as described (Givol et al., 1970). ^{32}P-labeled oligonucleotide P-2(a+b) (50 pmol in 5 μl H_2O; specific activity, 200 cpm/pmol) was allowed to run into a small column (0.1 ml) of immobilized RNAase T_1 and let stand for 15 min at room temperature. The RNA was rinsed out of the column and an aliquot was taken for electrophoretic analysis: only A-A-Gp, A-Gp, Gp and Up were detected. Digested RNA equivalent to 20 pmol of P-2(a+b) (as determined by radioactivity) was tested for inhibitory activity as described for the untreated fragments.

⧖ Free energy of optimal interaction with the sequence $_{HO}$A-U-U-C-C-U-C-C-A-C-U-A-G (5') of the 3' end of 16 S rRNA was calculated according to Gralla and Crothers (1973).

Fig. 9 Sedimentation analysis of complex formation between ribosomes and Qβ[^{3}H]RNA and [^{32}P]pA-U-G: effect of fMet-tRNA and oligonucleotide P-2(a+b). The reaction mixture contained 92 mM Tris-HCl (pH 7.5), 0.18 M NH_4Cl, 9.2 mM MgCl, 0.92 mM dithiothreitol. 0.92 mM GTP, 10 pmol of low salt-washed ribosomes, oligonucleotide as indicated below and about 1.3 nmol of stripped, unfractionated tRNA loaded with fMet (estimated to contain about 30 pmol fMet-tRNA) in a volume of 13 µl. After 15 min at 37 °C, 2 pmol of Qβ[^{3}H]RNA (o----o) (specific activity, 1.3 x 10^{5} cpm/pmol) and 100 pmol of [^{32}P] pA-U-G (● —— ●) (specific activity, 1.9 x 10^{4} cpm/pmol) were added in 7 µl and incubation was continued at 37 °C for 10 min. The sample was then analyzed essentially as described in Fig. 8. (a) No oligonucleotide, plus fMet-tRNA; (b) no oligonucleotide, no fMet-tRNA; (c) 40 pmol of P-2(a+b), plus fMet-tRNA. Brackets indicate the fractions corresponding to the 70 S complex.

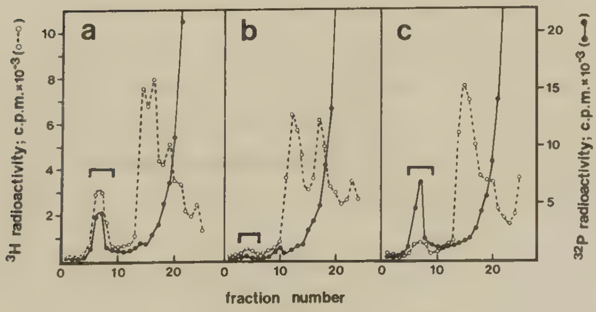
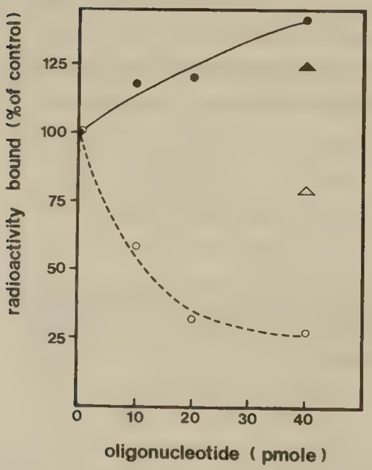


Fig. 10 Effect of oligonucleotide P-2(a+b) and P-6 on the binding of 70 S ribosomes to Qβ[^{3}H]RNA and [^{32}P] pA-U-G.

Qβ[^{3}H]RNA and [^{32}P]pA-U-G were incubated with ribosomes, fMet-tRNA and the amounts of P-2(a+b) or P-6 indicated and 70 S complex formation was determined as described in the legend to Fig. 9.

o ---- o, Qβ[^{3}H]RNA and P-2(a+b); ● —— ● [^{32}P]pA-U-G and P-2(a+b); Δ, Qβ[^{3}H]RNA and p-6; ▲, [^{32}P]pA-U-G and P-6.



At 10 pmol, the binding of A-U-G was 0.08 pmol, about one quater that of Qβ RNA at an input of 2 pmol. These result may indicate a different rate of complex formation for Qβ RNA and A-U-G triplet; it is less likely that the yield of 70 S complex would reflect an equilibrium between free and complexed components under the conditions of our assay, since centrifugation through a sucrose density gradient removes the excess oligonucleotide from the equilibrium. Oligonucleotide P-2a in mixture with P-2b inhibited binding of Qβ RNA but distinctly stimulated that of A-U-G (Fig. 9c and Fig. 10) at all levels tested; the values for 40 pmol of P-2(a+b) were 78 % inhibition for Qβ RNA and 42 % stimulation for A-U-G triplet. The corresponding values for 40 pmol of oligonucleotide P-6 (cf. Table 3) were 21 % and 25 %. The stimulation of binding of A-U-G triplet by P-2(a+b) may be due to increased availability of ribosomes which is no longer capable of binding to Qβ RNA.

Section III: Site-directed mutations in the initiator region of the Qβ coat cistron and their effect on ribosome binding

The results presented in section I demonstrated the absolute requirement of fMet-tRNA for the ribosome binding to true initiation site in the case of Qβ RNA. The question arose from this finding as to whether the initiator A-U-G triplet also plays a role for the formation of binding complex, and if so, to what extent.

One approach to assessing the role of the initiation triplet A-U-G for ribosome binding would be the use of mutants in which this sequence was modified. Classical procedures did

not appear adequate for obtaining such mutants, since, with the possible exception of the A-U-G → G-U-G transition, mutations of the initiation codon were expected to be highly disadvantageous or lethal.

Our laboratory has recently developed a procedure, site-directed mutagenesis, which permits the in vitro generation of nucleotide substitutions at predetermined positions of the Qβ genome. Minus strands are synthesized enzymatically in discrete steps starting at the 5' terminus. Elongation is controlled by the substrates offered at each step, and a nucleotide analog, N^4-hydroxyCMP, is introduced into the desired position of the minus strand. Utilization of such substituted minus strands as template for Qβ replicase gives rise to plus strands, 20 to 30 % of which have a nucleotide transition at the expected position. This procedure has been used to generate extracistronic mutations at the 40th and 16th positions from the 3' terminus of Qβ RNA, respectively (Flavell et al., 1974; Domingo et al., 1976; Sabo et al., 1977).

In this section, we report the preparation of Qβ RNA with G→A transitions of the third and fourth nucleotides of the coat cistron, i.e. with modifications in the third position of the A-U-G codon and the subsequent nucleotide. In order to introduce the nucleotide analog into the minus strand at the position corresponding to the beginning of the coat cistron, minus strand synthesis was resynchronized at a ribosome attached to the coat cistron initiation site (Kolakofsky et al., 1973). The minus strand was then elongated by stepwise synthesis, and the analog was inserted in the positions complementary to the third and fourth nucleotides of the coat cistron. The plus strand preparation resulting from the replication of these substituted minus strands contained the

Fig. 11 Nucleotide sequence of the replicase binding site (S-3) and the coat cistron ribosome binding site of Qβ RNA. The 3' proximal segment of the replicase binding site (straight arrow) overlaps with the 5' proximal part of the ribosome binding site (wavy arrow). Replicase binding and ribosome binding sites are defined as the RNA segments remaining attached to Qβ replicase (Weber et al., 1972) and ribosome (Hindley and Staples, 1969), respectively, after treatment of the binding complex with ribonuclease. Depending on the extent of the digestion a shorter (solid wavy arrow) or longer (dotted and solid arrow) RNA segment is retained on the ribosome.

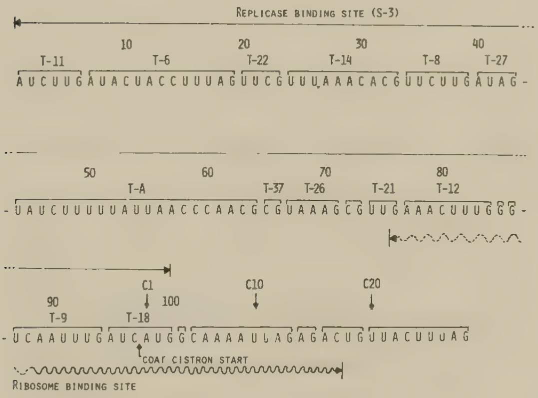
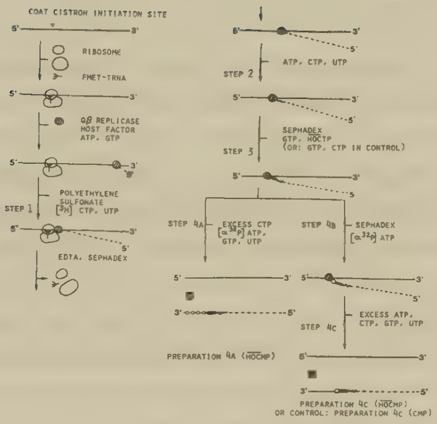


Fig. 12 Scheme for the introduction of N^4-hydroxyCMP into the Qβ minus strand at sites complementary to the 3rd and 4th nucleotides of the coat cistron.

A. Overview of the procedure. A ribosome is bound to Qβ RNA under initiation conditions and the 70 S ribosome-Qβ RNA complex is used as template for Qβ replicase. The minus strand is synthesized up to the point where the replicase runs into the ribosome (step 1). EDTA is added to dislodge the ribosome, and the replicating complex is chromatographed through Sephadex to remove nucleoside triphosphates and EDTA. Substrate-limited synthesis is carried out, using first ATP, UTP and CTP (step 2); GTP and the nucleotide analog are introduced in step 3. In a control experiment, GTP and CTP were added, instead. For analytical purposes, an aliquot of the replicating complex was passed through Sephadex and incubated with [α-^{32}P]ATP (step 4B), following which excess unlabeled ATP as well as the 3 other standard triphosphates were added to complete the minus strand. This specifically labeled preparation 4C ($\overline{\text{HOC}}$MP) (or 4C (CMP), in the case of the control) was analyzed to determine the extent of incorporation of the nucleotide analog into the desired position. For preparative purposes, the step 3 preparation was incubated with CTP in excess over the nucleotide analog and the other 3 nucleoside triphosphates; ATP was ^{32}P-labeled to allow the synthesis of the minus strand to be followed (preparation 4A ($\overline{\text{HOC}}$MP)).

B. Detailed view of stepwise synthesis. Top: The sequence of Qβ RNA at the beginning of the coat cistron is shown, and below it the sequence of the complementary minus strand segment. The wavy line indicates that segment of the minus strand synthesized when the ribosome is still bound to Qβ RNA (up to the "synchronization point"). The stepwise synthesis is described above.

A


COAT CISTRON INITIATION SITE
RIBOSOME
FMET-TRNA
Qβ REPLICASE
HOST FACTOR
ATP, GTP
POLYETHYLENE SULFONATE
STEP 1
[3H] CTP, UTP
EDTA, SEPHADEX
STEP 2
ATP, CTP, UTP
SEPHADEX
STEP 3
(OR: GTP, CTP IN CONTROL)
STEP 4A
EXCESS CTP
GTP, UTP
STEP 4B
SEPHADEX
STEP 4C
EXCESS ATP,
CTP, GTP, UTP
PREPARATION 4A (HOCMP)
PREPARATION 4C (HOCMP)
OR CONTROL: PREPARATION 4C (CMP)

B

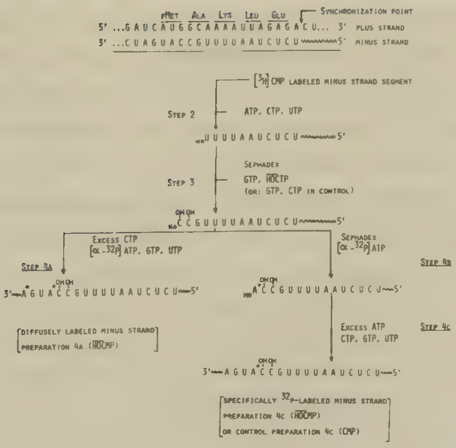

SYNCHRONIZATION POINT
PLUS STRAND
MINUS STRAND
CMP LABELED MINUS STRAND SEGMENT
STEP 2
ATP, CTP, UTP
SEPHADEX
STEP 3
(OR: GTP, CTP IN CONTROL)
EXCESS CTP
STEP 4A
SEPHADEX
STEP 4B
EXCESS ATP
CTP, GTP, UTP
STEP 4C
DIFFUSELY LABELED MINUS STRAND
PREPARATION 4A (HOCMP)
PREPARATION 4C (HOCMP)
OR CONTROL PREPARATION 4C (CMP)

Fig.12

expected mutant RNAs as well as wild-type RNA and was used for determining the relative ribosome binding capacity of the different species.

A) Insertion of N^4-hydroxyCMP into the Qβ minus strand positions complementary to the third and fourth nucleotide of the coat cistron.

The mutagenic nucleotide analog N^4-hydroxyCMP was introduced into the region of the minus strand complementary to the coat cistron ribosome binding site as outlined in Fig. 12.

Ribosomes were bound to Qβ RNA; as shown by Stamples & Hindley (1969), attachment occurs almost exclusively at the beginning of the coat cistron. The complex was isolated and used as template for Qβ replicase. Synthesis of the minus strand proceeded up to the position corresponding to the 16th nucleotide of the coat cistron (step 1) (Kolakofsky et al., 1973). Template utilization was about 12 %, not significantly lower than when uncomplexed Qβ RNA was used as template. The ribosome was then dislodged by treatment with EDTA, and the replicating complex (which is not affected by EDTA, cf. Kolakofsky et al., 1973) was incubated with ATP, CTP and UTP only, to allow elongation up to the position corresponding to the 5th nucleotide of the coat cistron (step 2). After removal of the unused substrates by Sephadex chromatography the replicating complex was incubated with GTP and N^4-hydroxyCTP (or, in a control experiment, with GTP and CTP), in order to insert the nucleotide analog (or CTP) into the positions complementary to the 4th and the 3rd nucleotides of the coat cistron (step 3). The preparation was then divided in two. One aliquot was incubated with an 120-fold excess of CTP over N^4-hydroxyCTP as well as

with all other substrates (the ATP being α-^{32}P-labeled to provide a measure for the elongation) to complete the minus strand (step 4A, preparation 4A ($H\overline{OC}MP$)). For analytical purposes the other aliquot was passed through a Sephadex column to remove unincorporated substrates and incubated with [α-^{32}P]ATP only (step 4B) in order to specifically label the last nucleotide inserted in step 3; the minus strands were then completed by adding an excess of unlabeled over labeled ATP as well as CTP, GTP and UTP (step 4C, preparations 4C ($H\overline{OC}MP$) and 4C (CMP) respectively). In several independent experiments the overall yield of purified substituted (or control) minus strands was 1.4 to 3.0 ng per µg of initial template.

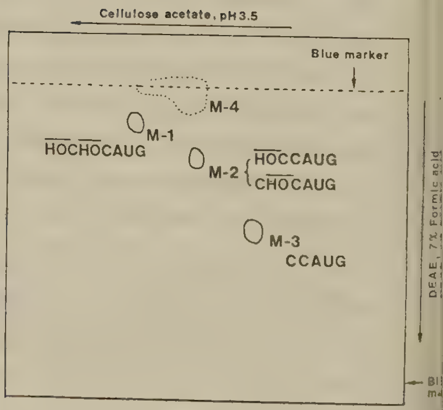
In order to determine whether the stepwise synthesis had proceeded in a sufficiently synchronized fashion, the minus strand preparations labeled with [^{32}P]AMP specifically at the site of insertion (step 4B) were digested with RNAase T_1, and the fragments separated by two-dimensional electrophoresis. The fingerprint of the control preparation 4C (CMP), which had been synthesized with CTP and GTP in the 3rd step, showed one spot with the same mobility as the oligonucleotide T_{50}, C-C-A-U-G, isolated by Kolakofsky et al. (1973) after a similar synthesis, and yielded CMP as the only labeled nucleotide after RNAase T_2 digestion. We may thus conclude that during the third stop CMP has been incorporated predominantly in the positions complementary to the third and fourth nucleotides of the coat cistron, as expected (cf. Fig. 12b).

The fingerprint of the ^{32}P-labeled minus strand containing $H\overline{OC}MP$ (preparation 4C ($H\overline{OC}MP$)) showed 3 major welldefined spots (M-1 to M-3) (Fig. 13). Oligonucleotide M-3 was identical with T_{50} as judged by its mobility relative to the

Fig. 13 Fingerprint of specifically labeled minus strands containing $\overline{\text{HOC}}$MP in the positions corresponding to nucleotide 3 and/or 4 of the coat cistron.
The synthesis of minus strands with insertion of $\overline{\text{HOC}}$MP in step 4A and [^{32}P]AMP in step 4B is described in the Materials and Methods section.
(a) A portion of the [^{32}P]AMP-labeled minus strand preparation 4C ($\overline{\text{HOC}}$MP)(8000 cts/min, 20 μg total RNA) was digested with 20 units of RNAase T_1 for 30 min at 37 °C. The digest was analyzed by two-dimensional electrophoresis following the procedure of Sanger & Brownlee (1967). (a') Tracing of (a) with identification of spots (cf. Table 4).

a

a'



blue dye marker and by the fact that it yielded [^{32}P] CMP after digestion with pancreatic RNAase (cf. Tabel 4). The fact that beside [^{32}P] CMP also 0.25 mol of [^{32}P] GMP were found suggests that in the second step some of the RNA chains were elongated up to the UMP residue complementary to the first nucleotide of the coat cistron (cf. Fig. 12b), due to contamination by GTP (derived from the first step). In the third step, these chains were elongated only by a GMP residue, which was then labeled by the [α-^{32}P]ATP added in step 4. The labeling pattern of oligonucleotide M-3 would thus be CpC$\overset{*}{\text{p}}$ApUpGp and CpCpApUp-G$\overset{*}{\text{p}}$(A). Oligonucleotide M-1 yielded [^{32}P]$\overline{\text{HOC}}$MP and traces of [^{32}P]GMP as only products after digestion with pancreatic RNAase; its mobility relative to oligonucleotide M-3 was greater in the first dimension and smaller in the second, about as expected for a nucleotide of the composition (U_{2-3}, A, G). Since $\overline{\text{HOC}}$MP has a mobility similar to that of UMP, we tentatively concluded that oligonucleotide M-1 had the structure $\overline{\text{HOC}}$p$\overline{\text{HOC}}\overset{*}{\text{p}}$ApUpGp(A). The labeled product M-2, with a mobility intermediate between M-1 and M-3, yielded both [^{32}P]$\overline{\text{HOC}}$MP and [^{32}P]CMP and only traces of [^{32}P]GMP, suggesting that it consisted of a mixture of Cp$\overline{\text{HOC}}\overset{*}{\text{p}}$ApUpGp(A) and $\overline{\text{HOC}}$pC$\overset{*}{\text{p}}$ApUpGp(A). Beside these three major, well-defined spots, the fingerprint showed a number of weaker spots and a diffusely darkened area M-4. The material eluted from the diffuse area M-4 yielded a large variety of ^{32}P-labeled products on digestion with pancreatic RNAase but no [^{32}P] $\overline{\text{HOC}}$MP. It thus seemed to consist of an undefined number of large oligonucleotides, most probably labeled in step 4C, when $\overline{\text{HOC}}$TP was no longer available as substrate. Since the specific activity of the

TABLE 4

Analysis of radioactive T_1 oligonucleotides from minus strands synthesized with N^4-hydroxyCTP in the third step and with [α-^{32}P] ATP in the fourth step+

Spot	Yield (cts/min)	Products of pancreatic RNAase digestion+ (mol[^{32}P])		Identification‡
M-1	410	$\overline{\mathrm{HOC}}\overset{*}{\mathrm{p}}$	(1)	$\overline{\mathrm{HOC}}\mathrm{p}\overline{\mathrm{HOC}}\overset{*}{\mathrm{p}}\mathrm{ApUpG}\overset{(*)}{\mathrm{p}}\mathrm{(Ap)}$
		$\mathrm{G}\overset{*}{\mathrm{p}}$	(0.04)	
M-2	600	$\overline{\mathrm{HOC}}\overset{*}{\mathrm{p}}$	(1)	$\overline{\mathrm{HOC}}\mathrm{pC}\overset{*}{\mathrm{p}}\mathrm{ApUpG}\overset{(*)}{\mathrm{p}}\mathrm{(Ap)}$ +
		$\mathrm{C}\overset{*}{\mathrm{p}}$	(1.9)	$\mathrm{Cp}\overline{\mathrm{HOC}}\overset{*}{\mathrm{p}}\mathrm{ApUpG}\overset{(*)}{\mathrm{p}}\mathrm{(Ap)}$
		$\mathrm{G}\overset{*}{\mathrm{p}}$	(0.02)	
M-3	470	$\mathrm{C}\overset{*}{\mathrm{p}}$	(1)	$\mathrm{CpC}\overset{*}{\mathrm{p}}\mathrm{ApUpG}\overset{(*)}{\mathrm{p}}\mathrm{(Ap)}$
		$\mathrm{G}\overset{*}{\mathrm{p}}$	(0.25)	
M-4	1340	Several products but no $\overline{\mathrm{HOC}}\overset{*}{\mathrm{p}}$		Mixture of large T_1 oligonucleotides

+ The minus strands were prepared as described in Materials and Methods. After digestion with RNAase T_1 the oligonucleotides were separated by two-dimensional electrophoresis on cellulose acetate and DEAE-paper, and analyzed as described in the legend to Fig. 3 and in Materials and Methods.

‡ The identification is based on the products of digestion with pancreatic RNAase and previous knowledge of the nucleotide sequence in this region of the minus strand (Kolakofsky et al., 1973). The wavy line indicates the part of the sequence labeled during the stepwise synthesis.

[^{32}P]ATP added after step 3 was diluted only 375-fold prior to elongating the minus strands to completion, almost all T_1 oligonucleotides should be slightly labeled; a variety of A-rich oligonucleotides accumulating in the same region of the fingerprint could account for the radioactivity in area M-4.

The analysis of oligonucleotides M-2 and M-3 indicated that traces of CTP were present during the third step of synthesis, either as contaminants of the $\overline{\text{HOC}}$TP preparation, or carried over from the previous step. Since CTP is incorporated in great preference to its analogue by Qβ replicase (Flavell et al., 1974) a very small contamination could account for these results.

From the analysis described above one may conclude that four species of minus strand were synthesized, differing in their structure in the region complementary to the third and fourth nucleotide of the coat cistron. Within the limitations of our analysis there was no evidence that substituation had occurred elsewhere. As summarized in Table 4, the relative amounts of the minus strand species with regard to their characteristic sequence were estimated to be as follows: $\overline{\text{HOC}}$-$\overline{\text{HOC}}$-A-U-G, 29 %; C-$\overline{\text{HOC}}$-A-U-G, 15 % $\overline{\text{HOC}}$-C-A-U-G, 29 %; C-C-A-U-G, 27 %. The non-statistical distribution of products cannot be accounted for by a simple explanation.

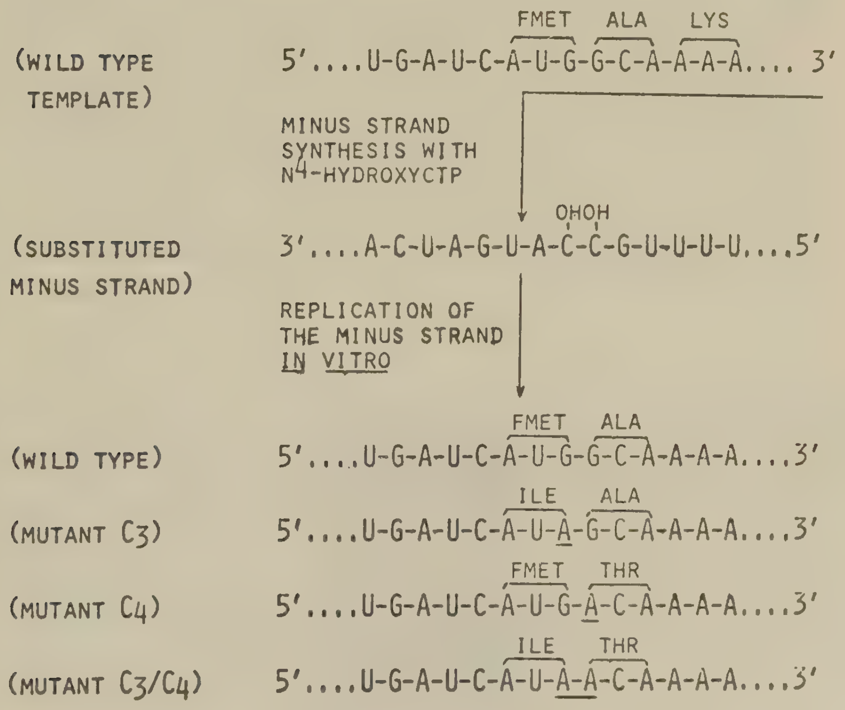
B) Analysis of plus strands synthesized with substituted minus strands as template.

The preparation containing $\overline{\text{HOC}}$MP subsituted minus strands (preparation 4A ($\overline{\text{HOC}}$MP)) was purified free of plus strands and replicated extensively by Qβ replicase. In a typical experiment, 0.4 µg of the minus strand gave rise to 6.4 µg of high molecular weight (30 S) RNA (preparation A) (cf. legend to Table 8).

It has been shown that N^4-hydroxyCMP, replacing either CMP or UMP at a unique position of the Qβ minus strand, directed the insertion of either GMP or AMP at the corresponding position of the plus strand, thereby giving rise to a transition mutation in part of the progeny RNA (Flavell et al., 1974; Domingo et al., 1977). Use of the preparation of substituted minus strands described above as template for replicase should therefore give rise to three species of mutant RNA as well as of wild type RNA, as illustrated in Fig. 14. In order to determine to what extent the expected mutations had been generated, the relevant region of Qβ RNA was subjected to nucleotide sequence analysis. For this purpose, two methods were applied: (i) isolation of the appropriate RNA segment by replicase binding (Weber et al., 1972) followed by classical fingerprinting analysis; (ii) total T_1 fingerprint analysis of the plus strand by two-dimensional polyacrylamide gel electrophoresis (De Wachter & Fiers, 1972).

a) Isolation and analysis of the replicase binding site "S". Qβ replicase binds mainly to two internal regions of Qβ RNA, to the M-site (located within the first third of the replicase cistron) and to the S-site, which precedes the coat cistron and comprises part of the ribosome binding site (Weber et al., 1972; Meyer et al., 1976). Exclusive binding to the S-site can be attained in 0.15 M NaCl, in the absence of Mg^{++}. After treatment of this Qβ replicase-Qβ RNA complex with RNAase T_1 a number of fragments, all derived from the S-site, can be isolated. The largest of these, the S-3 fragment, is 100 nucleotides long, comprises the intercistronic region preceding the coat cistron and extends up to (and includes) to initiator A-U-G codon of coat cistron (Weber et al., 1972) (cf. Fig. 11).

Fig. 14 The site-directed nucleotide substitutions at the coat cistron ribosome binding site of Qβ RNA.
Introduction of N^4-hydroxyCMP into specific positions of the minus strand was carried out as shown in Fig.12. Since N^4-hydroxyCMP directs the incorporation of AMP or GMP, four species of plus strands are expected.



Since the expected mutations affect only the last nucleotide of the 100 nucleotide-long segment, this isolation method is expected to yield a representative sample of the S-sites of the plus strand population. As shown below, this allows the quantitative analysis, by nearest neighbor labeling, of the region comprising nucleotides C-3 and C-4.

Highly radioactive [α-^{32}P]AMP-labeled RNA was synthesized, using as template on the one hand the RNA preparation "A" described above, and on the other, wild type Qβ RNA as control. Qβ replicase was bound to the labeled RNA preparations and the fragments retained by the enzyme after treatment with RNAase T_1 were subjected to electrophoresis through a 20 % polyacrylamide gel. Whereas [^{32}P]AMP-labeled Qβ RNA synthesized with wild type RNA as template yielded mainly one strong band, namely S-3, the RNA derived from the substituted minus strands showed three strong bands: S-3', with the mobility of S-3, a slower one, designated S-3* and a faster one, S-3** (Fig. 15). The fragments S-3' and S-3* were subjected to T_1 fingerprint analysis (Fig. 16). S-3' (Fig. 16b) showed a pattern identical to that of S-3 (Fig. 6a) from wild type RNA; each T_1 oligonucleotide except for Q-18) was found to be identical with a wild type counterpart sequenced by Weber et al. (1972), as judged by nearest neighbor analysis (Table 5) and mobility. Spot Q-18, which had the same mobility as T-18 of the wild type, was heterogeneous. T-18 is the 3' terminal T_1 oligonucleotide of the S-site and has the sequence A-U-C-A-U-G(G) (Weber et al., 1972). It is thus derived from the region in which the site-directed mutations are expected to occur (cf. Fig. 11 and legend to Table 5). Upon RNAase A digestion of the heterogeneous [^{32}P]AMP-labeled product Q-18 three radioactive oligonucleotides were recovered in non-equimolar

Fig. 15 Electrophoretic analysis of Qβ[^{32}P]RNA fragments from wild type and mutant Qβ RNA bound to Qβ replicase after RNAase T_1 digestion.
Qβ[^{32}P]RNA was incubated with Qβ replicase under "S-site" binding conditions, the mixture was treated with RNAase T_1 and the enzyme-bound RNA recovered. After ethanol precipitation, RNA was dissolved in 20 µl of 0.5 mM EDTA and denatured by heat before loading on a 20 % polyacrylamide gel. The gel was run at 750 V for about 20 h and an autoradiograph was prepared. For details, see Materials and Methods.
(a) Replicase binding site fragment from [^{32}P]AMP-labeled RNA (1.9 x 10^7 cts/min) synthesized in vitro on wild type Qβ RNA; (b) replicase binding site fragment from [^{32}P]AMP-labeled RNA (5.7 x 10^7 cts/min) synthesized in vitro on hydroxyCMP-substituted Qβ minus strands.

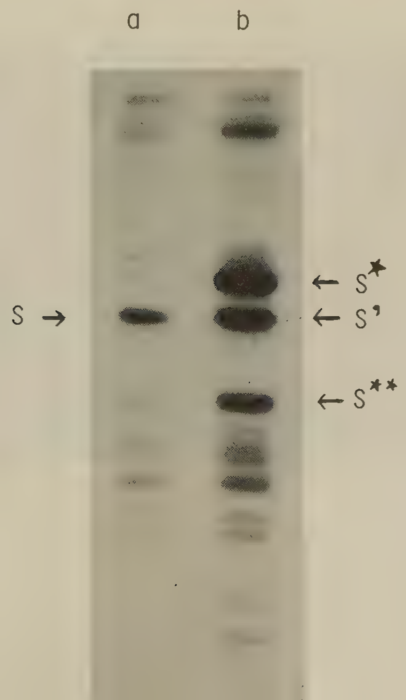
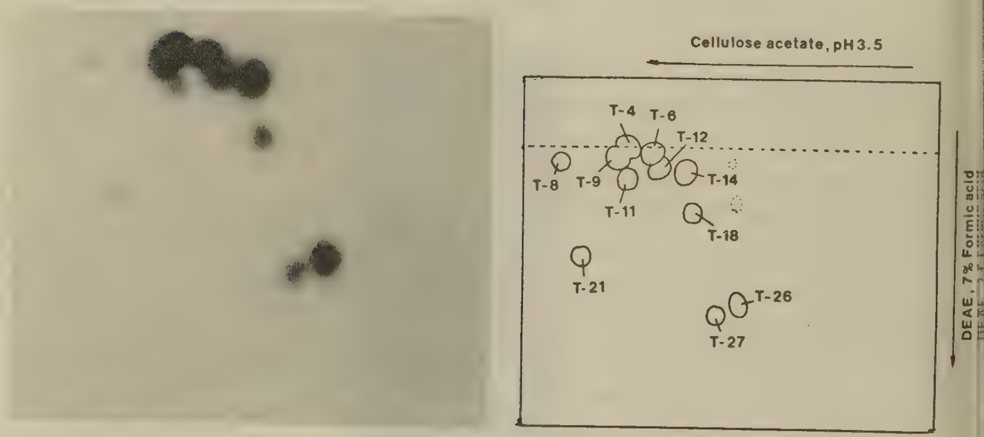


Fig. 16 Fingerprint of replicase-bound fragments purified from a mutant RNA preparation.

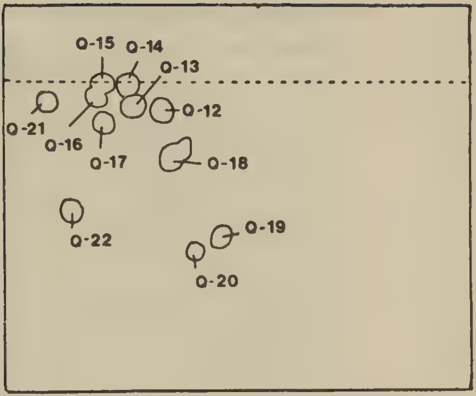
The replicase-bound RNA segments designated as S-3' (1.5×10^4 cts/min) and S-3* (2.6×10^4 cts/min) in Fig. 15b were eluted from the gel, mixed with 20 µg of carrier RNA, digested with 10 units of RNAase T_1 and analyzed by two-dimensional electrophoresis, as detailed in the Materials and Methods section. (a) Autoradiogram of T_1 digests of wild type S-3 (2.0×10^4 cts/min); (a') tracing of (a); (b) autoradiogram of T_1 digests of S-3'; (b') tracing of (b); (c) autoradiogram of T_1 digests of S-3*; (c') tracing of (c). The correlation of the oligonucleotides and their sequence are given in Table 5.

a a'

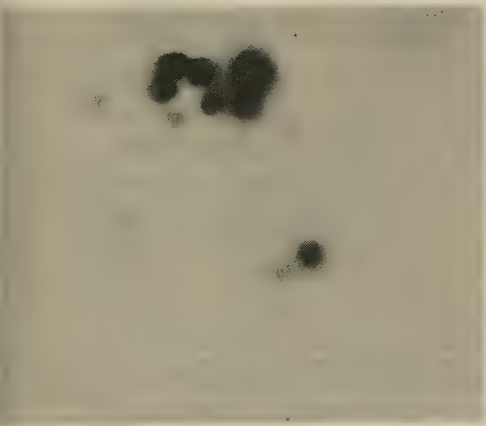


b

b′


Q-15
Q-14
Q-13
Q-12
Q-21
Q-16
Q-17
Q-18
Q-22
Q-19
Q-20

c



c′

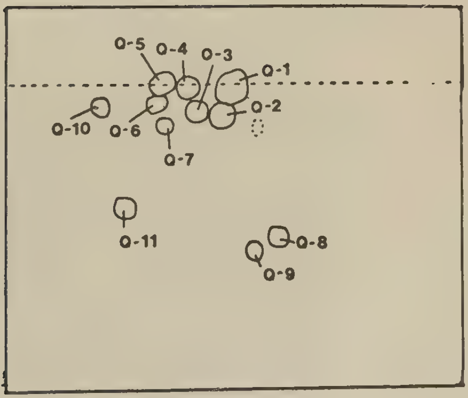

Q-5
Q-4
Q-3
Q-1
Q-2
Q-10
Q-6
Q-7
Q-11
Q-8
Q-9

TABLE 5

Analysis of the T_1 oligonucleotides from the replicase binding site S of a mutant Qβ RNA preparation labeled with [α-^{32}P] ATP †

Fragment	Oligo-nucleotide	Pancreatic digestion products (mol ^{32}P)$^{+}$	Interpretation‡ (nomenclature by Meyer & Weber, pers.comm.)		Yields (cts/min)	Molar yield^{+}
S-3*	Q-1	Ap*Ap*Ap*ApUp(3.5); Ap*ApCp*(2.2) Ap*Gp(1); Ap*Up(1.0); Cp*(0.9) Up* (1.4)	AUCAUAACAAAAUUAG(A)	-	2910	0.94
	Q-2	Ap*Ap*ApCp*(3.1); Up*(1)	UUUAAACACG(U)	(T-14)	1380	1
	Q-3	Ap*Ap*ApCp	AAACUUUG(G)	(T-12)	700	1.01
	Q-4	ApUp*(1); Up*(2.1)	AUACUACCUUUAG(U)	(T-6)	940	0.91
	Q-5	Ap*ApCp(1.7); Cp*(1.0) Up*(2.6)	UAUCUUUUUAUUAACCCAACG(C)	(T-4)	2110	1.02
	Q-6	Ap*ApUp(1.0); Gp*(1) Cp*(0.9)	UCAAUUUG(A)	(T-9)	1000	0.97
	Q-7	Gp*	AUCUUG(A)	(T-11)	320	0.93
	Q-8	Ap*Ap*ApGp(2.1); Up*(1)	UAAAG(C)	(T-26)	1020	0.99
	Q-9	ApUp*	AUAG(U)	(T-27)	390	1.13
	Q-10	Gp*	UUCUUG(A)	(T-8)	350	1.01
	Q-11	Gp*	UUG(A)	(T-21)	310	0.90
S-3'	Q-12	Ap*Ap*ApCp*(2.8); Up*(1)	UUUAAACACG(U)	(T-14)	1000	1
	Q-13	Ap*Ap*ApCp	AAACUUUG(G)	(T-12)	480	0.96
	Q-14	ApUp*(1); Up(3.0)	AUACUACCUUUAG(U)	(T-6)	720	0.96
	Q-15	Ap*ApCp(2.1); Cp*(1) Up*(2.9)	UAUCUUUUUAUUAACCCAACG(C)	(T-4)	1190	0.79
	Q-16	Ap*ApUp(1.0); Gp*(1) Cp*(1)	UCAAUUUG(A)	(T-9)	740	0.99
	Q-17	Gp*	AUCUUG(A)	(T-11)	250	1.00
	Q-18 { Q-18a		AUCAUG(G)	(T-18)		0.35 }
	Q-18 { Q-18b	ApUp*(0.3); Gp*(0.4)	AUCAUG(A)	-	500	0.47 } 1.17
	Q-18 { Q-18c	Cp*(1)	AUCAUAG(C)	-		0.35 }
	Q-19	Ap*Ap*ApGp(2.1); Up(1)	UAAAG(C)	(T-26)	790	1.05
	Q-20	ApUp*	AUAG(U)	(T-27)	250	1.00
	Q-21	Gp*	UUCUUG(A)	(T-8)	190	0.76
	Q-22	Gp*	UUG(A)	(T-21)	240	0.96

†[^{32}P]AMP-labeled RNA was synthesized in vitro with Qβ replicase under standard conditions using 0.4 μg of substitutes minus strands and [α-^{32}P]ATP with a specific activity of 3 x 10^4 cpm/nmol. 30 S RNA was selected by sucrose gradient centrifugation. Qβ replicase was bound to the RNA in the presence of 0.15 M NaCl and in the absence of Mg^{++}. The resulting complex was treated with RNAase T_1, collected on a Millipore filter and the [^{32}P]RNA was extracted. After electrophoresis through a 20% polyacrylamide gel (cf. Fig.15) the fragments S-3' and S-3* were eluted and subjected to T_1 fingerprint analysis (see Materials and Methods for details).

$^{+}$Yields are expressed relative to the underlined value.

‡Based on the pancreatic digestion products, the mobilities compared to those of the T_1 products described by Weber et al. (1972), the known sequence of the S binding site and the assumption that the mutant RNA species arose by G → A transitions. The proposed sequences in the 3' terminal region are as follows

Sequence		
AUCU.....GUCAAUUUGAUCAUG(G) [Q-16=Q-6, Q-18a]	wild type	S-3'
AUCU.....GUCAAUUUGAUCAUAG(C) [Q-18c]	mutant C_3	S-3'
AUCU.....GUCAAUUUGAUCAUG(A) [Q-18b]	mutant C_4	S-3'
AUCU.....GUCAAUUUGAUCAUAACAAAAUUAG(A) [Q-1]	mutant C_3/C_4	S-3*

yield; $\mathrm{C\overset{*}{p}}$ (1), $\mathrm{G\overset{*}{p}}$ (0.4) and $\mathrm{ApU\overset{*}{p}}$ (0.3), whereas T-18 from wild type plus strands released $\mathrm{C\overset{*}{p}}$ as only radioactive product (result not shown). Assuming that the oligonucleotides present in spot Q-18 are identical with T-18 or derived from it by a single nucleotide transition, $\mathrm{G\overset{*}{p}}$ must have originated from $\mathrm{ApUpC\overset{*}{p}ApUpG\overset{*}{p}(A)}$, i.e. the sequence resulting from a G → A transition in the 4th position of the coat cistron (C_4), and $\mathrm{ApU\overset{*}{p}}$ from $\mathrm{ApUpC\overset{*}{p}ApU\overset{*}{p}ApGp(C)}$, i.e. the sequence due to a G → A transition in the 3rd position of the cistron (C-3). $\mathrm{C\overset{*}{p}}$ originated from wild type as well as from mutant sequences. The heterogeneity of Q-18 thus most likely reflects the presence of two oligonucleotides differing by one A residue, with the wild type oligonucleotide sequence occurring in two different labeling patterns. The analysis of Table 5 allows the quantitation of wild type and mutant C-3 and C-4 RNAs by using the following relationships:

$$\text{wild type} = [\mathrm{C\overset{*}{p}}] - [\mathrm{ApU\overset{*}{p}}] - [\mathrm{G\overset{*}{p}}] \simeq 0.3$$

$$\text{mutant C3} = [\mathrm{ApU\overset{*}{p}}] \simeq 0.3$$

$$\text{mutant C4} = [\mathrm{G\overset{*}{p}}] \simeq 0.4$$

This analysis did not reveal RNA carrying mutations in C3 as well as in C4 (mutant RNA C3/C4), since, as will be shown below, this species does not yield the usual S-3 fragment but a longer sequence, S-3*.

The T_1 fingerprint of the S-3* fragment (Fig. 16c) showed all oligonucleotides found in S-3, with exception of oligonucleotide Q-18, which was replaced by Q-1. Digestion of $[^{32}P]$AMP-labeled Q-1 by pancreatic RNAase yielded, among other products $\mathrm{A\overset{*}{p}A\overset{*}{p}A\overset{*}{p}ApUp}$, $\mathrm{U\overset{*}{p}}$ and $\mathrm{ApG\overset{*}{p}}$, which as can be seen from Fig. 11, are most likely derived from the sequence following the normal 3' terminus of S-3. In addition, oligonucleotide Q-1 gave rise to $\mathrm{C\overset{*}{p}}$, $\mathrm{ApU\overset{*}{p}}$ and $\mathrm{A\overset{*}{p}AC\overset{*}{p}}$,

suggesting that it had the structure ApUpC$\overset{*}{p}$ApU$\overset{*}{p}$$\underline{A\overset{*}{p}A\overset{*}{p}}C\overset{*}{p}A\overset{*}{p}$-A$\overset{*}{p}A\overset{*}{p}$ApUpU$\overset{*}{p}ApG\overset{*}{p}$(A). This is the sequence expected in place of T 18 if two G → A transitions had occurred in positions 3 and 4 respectively of the coat cistron (underlined above) (cf. Fig. 11). The proposed structure of Q-1 was supported by additional nearest neighbor data given in Table 6. This interpretation also explains why part of the replicase-RNA complexes, on T_1 RNAase digestion, yielded a new fragment S-3*; as a consequence of the replacement of the two GMP residues by AMP, the normal cleavage site was eliminated and scission took place at the closest unprotected GMP residue to the 3' side, at position C-13 (cf. Fig. 11). Oligonucleotide S-3** was not analyzed because it was usually present in low yield only. The other, minor bands visible in Fig. 15, as far as they were analyzed, proved to be fragments of S-3' or S-3* (data not shown).

b) Analysis by two-dimensional polyacrylamide gel electrophoresis. In order to determine the content of mutant C_3/C_4 RNA, the [^{32}P]AMP-labeled RNA preparation described above was purified free of minus strands, digested completely with RNAase T_1 and subjected to two-dimensional polyacrylamide gel electrophoresis. This procedure displays about 30 large T_1 oligonucleotides of 12 to 26 residues (M.A. Billeter, personal communication). The plus strand RNA derived from the substituted minus strands gave a pattern identical to that of wild type RNA, except for a reduction of spot 12 and an additional oligonucleotide (arrow, Fig. 17b). Oligonucleotide T-12 is derived from a position about 1000 nucleotides downstream from the coat cistron ribosome binding site (M.A. Billeter, personal communication); its reduction is most likely due to an incidental mutation which occurred during the *in vitro* amplification of the RNA (Sabo et al., 1977).

Fig. 17 Two-dimensional polyacrylamide gel electrophoresis analysis of the T_1 oligonucleotides of wild type or "mutant" plus strands.
[^{32}P]AMP-labeled mutant and wild type plus strands were purified as described in Materials and Methods from the same RNA preparations described in the legend to Fig.16. Each preparation (1,5 x 10^6 cts/min wild type; 5.6 x 10^6 cts/min "mutant" plus strands) contained 80 µg of unlabeled Qβ RNA as carrier and was digested with 40 units of RNAase T_1 in 20 mM-Tris-HCl (pH 7.5), 2 mM-EDTA (final volume, 10 µl) for 30 min at 37 °C. Two-dimensional polyacrylamide gel electrophoresis was carried out as described by De Wachter & Fiers (1972), using a 10% gel in the first and a 20% gel in the second dimension. (a) Autoradiogram of the T_1 digest of wild type plus strands; (a') tracing of (a); (b) autoradiogram of T_1 digest of "mutant" plus strands; (b') tracing of (b). The numbering is that of Billeter (personal communication).
For the quantitation of mutant C_3/C_4, the molar yield of oligonucleotide Q-1 was obtained by comparing the radioactivity per labeled nucleotide of oligonucleotides Q-1 (1040 cts/min), T-2 (2270 cts/min), T-6 (2350 cts/min) and T-15 (2000 cts/min).

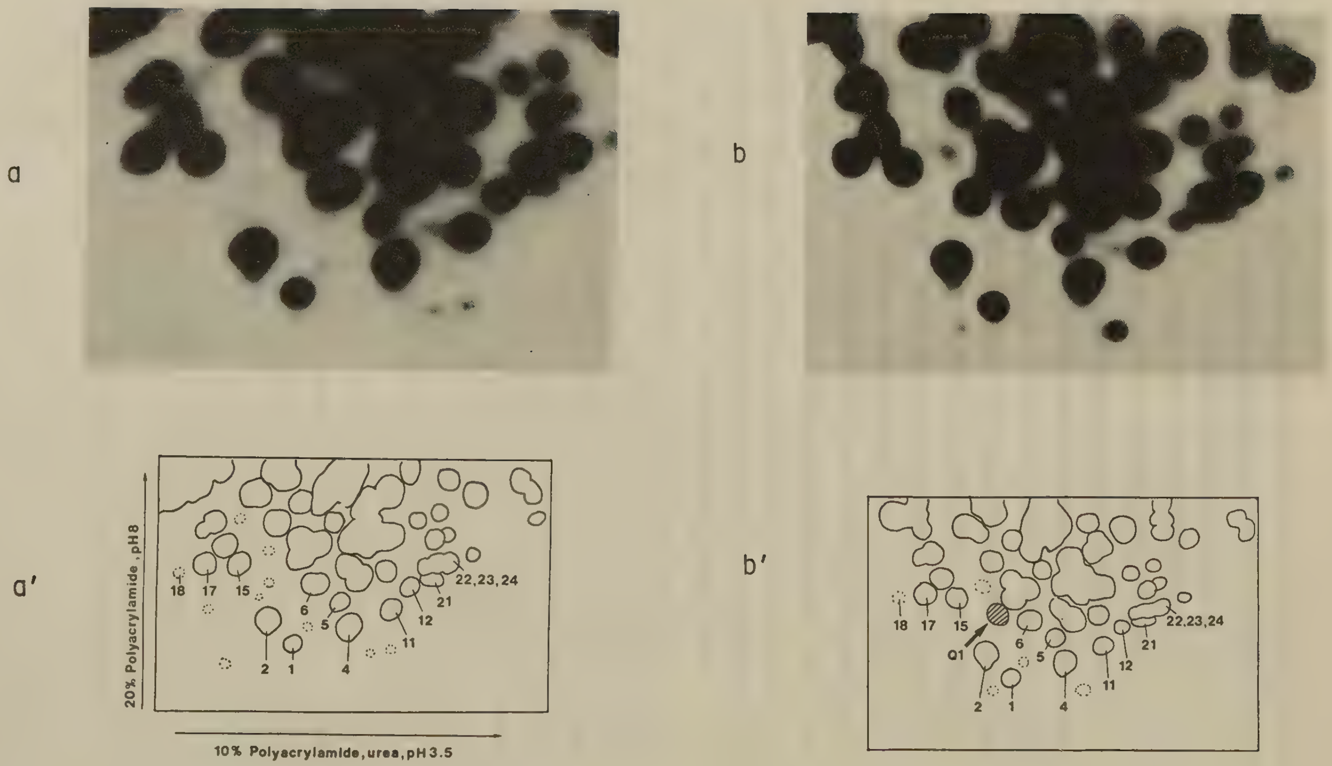

a
b
a′
18
17
15
6
5
2
1
4
11
12
21
22,23,24
20% Polyacrylamide, pH8
10% Polyacrylamide, urea, pH3.5
b′
Q1

Fig.17

The new oligonucleotide, on digestion with RNAase A, gave the same products as Q-1, A-U-C-A-U-A-A-C-A-A-A-A-U-U-A-G-(A), characteristic for the C_3/C_4mutant (Table 6). The proportion of mutant C_3/C_4RNA, as judged by the molar yield of oligonucleotide Q-1, was about 47% of the total RNA population (cf. legend to Fig. 17). From this number and the data of the previous section (which gives the ratio of wild type : mutant C_3 : mutant C_4) the ratio of wild type : mutant C_3 : mutant C_4 : mutant C_3/C_4 was calculated to be 1 : 1 : 1.3 : 2.9.

The finding of the C_3/C_4 mutant RNA in such a large proportion was unexpected, since (a) this mutant originated from minus strands with two HOCMP substitutions which constituted not more than 30% of the minus strands preparation and (b) G → A transitions induced by the analog occur with less than 50% frequency (Flavell et al., 1974). It has been shown that Qβ RNA carrying an extracistronic mutation at the 16th position from its 3' end (Sabo et al., 1977) is preferentially replicated by Qβ replicase *in vitro*, while a transition in the 40th position from the 3' end retards *in vitro* replication substantially (Domingo et al., 1976). In view of the substantial effects a single nucleotide change may have in regard to replication, and considering that the mutations we are dealing with are located close to or in an RNA region which interacts with Qβ replicase (Weber et al., 1972), it is very likely that the C_3/C_4 mutant RNA was originally present in a low proportion but that, due to selective advantage as template, it was preferentially replicated during *in vitro* amplification. In several other RNA preparations, some carried out with other enzyme batches, the ratio of mutant C_3/C_4 to wild type varied from 0.24 to 5.

C) Ribosome binding capacity of Qβ RNA with G → A transitions in the third and fourth nucleotides of the coat cistron. As shown in Fig. 11, positions C-3 and C-4, in which G → A transitions were generated, lie within the ribosome binding site of the coat cistron. In order to determine the relative ribosome binding capacity of the different species of Qβ RNA with and without the G → A transitions, an excess of ribosomes was bound to a preparation of full-length [^{32}P] AMP-labeled RNA consisting of the 3 mutants C_3, C_4 and C_3/C_4 and the wild type species in known proportions (cf. section b or Table 8). The preparation also contained about 40% of the corresponding minus strands; these had not been removed since they were known not to bind to ribosomes (Feix et al., 1968; Schwarz et al., 1969). The 70 S complexes, comprising about 17% of the input radioactivity were isolated and treated with pancreatic RNAase; the ribosome-bound radioactive RNA (about 1% of input) was recovered, and purified by polyacrylamide gel electrophoresis. Fig. 18 shows that the ribosome bound RNA consisted predominantly of a single major band; it had the same mobility as the coat cistron ribosome binding site isolated from wild type RNA (data not shown). The material contained in the main band was digested with RNAase T_1 and the products were separated by 2-dimensional electrophoresis (Fig. 19a). Fig. 19b shows the fingerprint of the ribosome binding site obtained from (homogeneously labeled) wild type RNA. It is apparent that the mutant RNA yielded one oligonucleotide, R-2, not given by the wild type RNA and that R-1 is more diffuse than its wild type counterpart. Each T_1 oligonucleotide was identified by its mobility and/or the products it yielded upon digestion with pancreatic RNAase (Table 7).

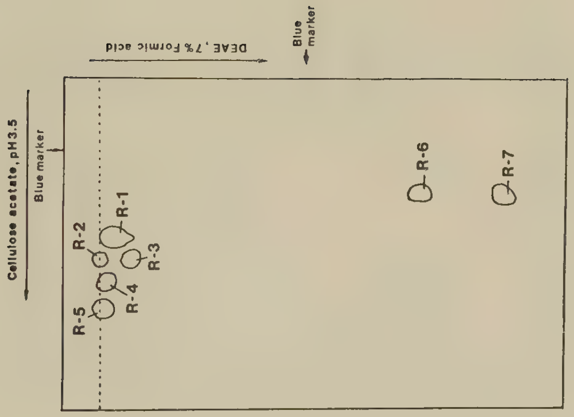
Oligonucleotide R-2, present in 0.12 molar yield, was identical with Q-1, and was therefore derived from the

Fig. 18 Electrophoretic analysis of the ribosome-bound RNA isolated from a "mutant" RNA preparation.
The [^{32}P]AMP-labeled RNA preparation is the same as the one used in the experiments described in the legends to Figs. 15 and 17. Ribosomes were bound to the RNA (1.5 x 10^8 cts/min), the 70 S complex was isolated, treated with pancreatic RNAase and the ribosome-bound RNA (38,000 cts/min) was subjected to polyacrylamide gel electrophoresis and autoradiography as described in the Materials and Methods section.

Fig. 19 (a) T_1 fingerprint of the purified coat cistron ribosome binding site isolated from a "mutant" RNA preparation. The isolation and purification of ribosome-bound RNA from a [^{32}P]AMP-labeled "mutant" RNA preparation was described in the legend to Fig. 18. The major band appearing on the polyacrylamide gel shown in Fig. 18 (5,000 cts/min) was eluted, digested with RNAase T_1, analyzed by two-dimensional electrophoresis on cellulose acetate and DEAE cellulose and autoradiographed as described in the Materials and Methods section. (a'), tracing of (a).

(b) T_1 fingerprint of purified coat cistron ribosome binding site isolated from uniformly ^{32}P-labeled natural Qβ RNA (3×10^7 cts/min). Isolation of the fragment and analysis were as described above; (b') tracing of (b); the identification of the oligonucleotides is from Hindley & Staples (1969), Weber et al. (1972).

a'


Cellulose acetate, pH3.5
Blue marker
DEAE, 7% Formic acid
Blue marker
R-1
R-2
R-3
R-4
R-5
R-6
R-7

a

Fig.19

b

b′

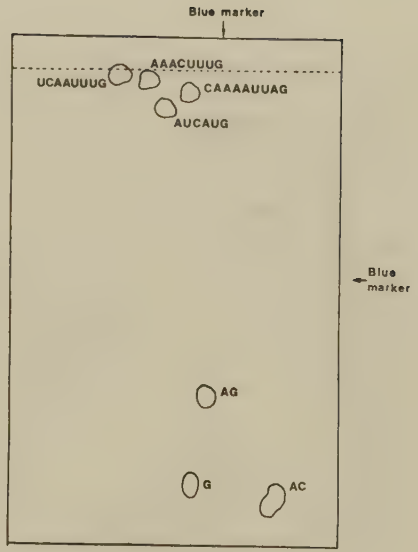

Blue marker
AAACUUUG
UCAAUUUG
CAAAAUUAG
AUCAUG
Blue marker
AG
G
AC

Fig.19

TABLE 7

Analysis of the T_1 oligonucleotides of the coat cistron ribosome binding site recovered from a preparation of [^{32}P]AMP-labeled mutant Qβ RNA*

Oligonucleotide		Pancreatic digestion product (mol ^{32}P)	Interpretation‡	Yield (cts/min)	Molar yield	
R-1	R-1a	Ap*Ap*Ap*ApUp(3.5);ApGp*(1);	CAAAAUUAG(A)	800	0.35	0.88
	R-1b	ApCp*(0.6);Cp(0.4);Up*(1.1)	ACAAAAUUAG(A)		0.53	
R-2	(= Q-1)+	Ap*Ap*Ap*ApUp(3.6);Ap*ApCp*(2.0); ApGp*(1.6);ApUp*(1);Cp*(0.9); Up*(1.2)	AUCAUAACAAAAUUAG(A)	160	0.12	
R-3	R-3a (= Q-18a)+	Gp*(0.7);Cp*(1)	AUCAUG(G)	230	0.26	0.89
	R-3b (= Q-18b)+		AUCAUG(A)		0.63	
R-4	(= Q-3 = Q-13)+	Ap*Ap*ApCp	AAACUUUG(G)	300	1	
R-5	(= Q-6 = Q-16)+	Ap*ApUp(1.0);Gp*(1) Cp*(1.1)	UCAAUUUG(A)	460	1.00	
R-6		ApGp*	AG(A)	160	1.10	
R-7		Gp*	G(A)	160	1.10	

TABLE 7 (cont.)

*The preparation was the same as the one analyzed in Table 5. Ribosomes were bound to the RNA and the resulting 70 S complex was isolated and treated with pancreatic RNAase. The protected RNA segment was extracted, purified by 20 % polyacrylamide gel electrophoresis and subjected to T_1 fingerprint analysis (for details, see Materials and Methods). Molar yields are relative to underlined items.

+See Table 5.

‡Based on the pancreatic digestion products, the mobilities compared to those of the T_1 products described by Staples. & Hindley (1969) and Weber et al. (1972), the known sequence of the coat binding site and the assumption that the mutant RNA species arise by G → A transitions.
The proposed sequences of the ribosome binding sites and Their proportions are as follows:

wild type	R-7 R-4 R-5 R-3a R-1a R-6 GAAACUUUGGGUCAAUUUGAUCAUGGCAAAAUUAGAGAC(U)	30 % (26-35%)
mutant C_3	not present R-1a GAAACUUUGGGUCAAUUUGAUCAUAGCAAAAUUAGAGAC(U)	< 10 %
mutant C_4	R-3b R-1b GAAACUUUGGGUCAAUUUGAUCAUGACAAAAUUAGAGAC(U)	58 % (53-63%)
mutant C_3/C_4	R-2 GAAACUUUGGGUCAAUUUGAUCAUAACAAAAUUAGAGAC(U)	12 %

RNA mutant C_3/C_4. The proposed structure, A-U-C-A-U-A-A-C-A-A-A-A-U-U-A-G(A), was supported by additional nearest neighbor data obtained from [^{32}P] UMP and [^{32}P] CMP-labeled products (cf. Table 6). The [^{32}P]AMP-labeled product(s) R-3 had the mobility of Q-18, which consisted of A-U-C-A-U-G and A-U-C-A-U-A-G (cf. Table 5) and, on pancreatic RNAase digestion, yielded 1 $\mathrm{C\overset{*}{p}}$ and 0.7 $\mathrm{G\overset{*}{p}}$ but no $\mathrm{AU\overset{*}{p}}$. This indicates a composition of 0.7 mol of $\mathrm{ApUpC\overset{*}{p}ApUpG\overset{*}{p}}$(A), derived from mutant C_4RNA, and 0.3 mol of $\mathrm{ApUpC\overset{*}{p}ApUpGp}$(G), derived from wild type RNA, and no $\mathrm{ApUpC\overset{*}{p}ApU\overset{*}{p}ApGp}$(C), characteristic for mutant C_3RNA. Taking into account that the relative yield of R-3 was 0.9, the proportion of C_4 RNA in the ribosome-bound RNA was 0.9 x 0.7 = 0.63.

The heterogeneous product R-1, present in 0.88 molar yield, gave the pancreatic digestion products $\mathrm{A\overset{*}{p}A\overset{*}{p}A\overset{*}{p}ApUp}$, $\mathrm{U\overset{*}{p}}$, $\mathrm{A\overset{*}{p}Gp}$ and 0.4 mol $\mathrm{C\overset{*}{p}}$, which are diagnostic for the oligonucleotide $\mathrm{C\overset{*}{p}A\overset{*}{p}A\overset{*}{p}A\overset{*}{p}ApUpU\overset{*}{p}ApGp}$(A) (R-1a), as well as 0.6 mole of $\mathrm{ApC\overset{*}{p}}$, which most likely is derived from the oligonucleotide A-C-A-A-A-**A**-U-U-A-G (R-1b) arising from the RNA with the C_4 mutation (cf. Table 6). These assignments were confirmed by nearest neighbor data obtained from RNA preparations labeled with [^{32}P] CMP and [^{32}P] UMP in later experiments (results not shown). From these data, the proportion of C_4 RNA bound to the ribosome was 0.53, in reasonable agreement with the value of 0.6 arrived at above. From the yields of the wild type oligonucleotides $\mathrm{ApUpC\overset{*}{p}ApUpGp}$(G) and $\mathrm{C\overset{*}{p}A\overset{*}{p}A\overset{*}{p}A\overset{*}{p}ApUpU\overset{*}{p}ApGp}$(A) the proportion of wild type RNA in the ribosome bound complex was estimated to be 0.26 to 0.35.

The experiment thus shows that when ribosomes were bound to an RNA preparation containing wild type, mutant C_3, mutant C_4 and mutant C_3/C_4 RNA in the ratio 1 : 1 : 1.3 : 2.9, the composition of the RNA bound to the ribosome was

TABLE 6

Characterization of the new large T_1 oligonucleotide Q-1 (= R-2) derived from mutant C_3/C_4.

$[\alpha-^{32}P]$NTP used for labeling	Method of obtaining fragment from which Q-1 was isolated	Pancreatic digestion products of oligonucleotide Q-1⁺ (mol$[^{32}P]$)
ATP	ribosome binding	$A\overset{*}{p}A\overset{*}{p}A\overset{*}{p}ApUp$(2.3); $A\overset{*}{p}ApC\overset{*}{p}$(1.3); $ApG\overset{*}{p}$(1); $ApU\overset{*}{p}$(0.6); $C\overset{*}{p}$(0.6); $U\overset{*}{p}$(0.8)
	replicase binding	$A\overset{*}{p}A\overset{*}{p}A\overset{*}{p}ApUp$(3.5); $A\overset{*}{p}ApC\overset{*}{p}$(2.2); $ApG\overset{*}{p}$(1); $ApU\overset{*}{p}$(1.0); $C\overset{*}{p}$(0.9); Up(1.4)
	total T_1 fingerprint	$A\overset{*}{p}A\overset{*}{p}A\overset{*}{p}ApUp$(3.1); $A\overset{*}{p}ApC\overset{*}{p}$(2.1); $ApG\overset{*}{p}$(1); $ApU\overset{*}{p}$(1.0); $C\overset{*}{p}$(1.1), $U\overset{*}{p}$(1.3)
UTP	ribosome binding	$ApApApA\overset{*}{p}U\overset{*}{p}$; $A\overset{*}{p}Up$‡
CTP	ribosome binding	$ApA\overset{*}{p}Cp$(1); $ApU\overset{*}{p}$(1.2)

⁺The data define the structure (A-U-C)(A-U)(A-A-C)(A-A-A-A-U-U)A-G(A). Assuming that the mutant sequence arises by two G → A transitions from the known wild type sequence of the ribosome binding site (cf. Fig.11), the structure of Q-1 is A-U-C-A-U-A-A-C-A-A-A-A-U-U-A-G(A).

‡The intensity of the spots on the autoradiogram was estimated visually.

wild type: mutant C_3 : mutant C_4 : mutant C_3/C_4 = 1 : < 0.1 : 1.9 : 0.4. Therefore, the mutant RNA C_4, in which the A-U-G triplet was conserved, was bound 1.5 times more efficiently than wild type RNA. The mutant RNA C_3/C_4, in which the initiation triplet had been converted to the sequence A-U-A, also bound to ribosomes, albeit with an efficientcy about 0.14 relative to that of wild type RNA. Mutant C_3, which also lacked the initiation triplet, did not bind to a detectable extent, i.e., with less than 5% to 10% efficiency.

The complete experiment was repeated twice, starting with an independent preparation of substituted minus strands and a different ribosome preparation (Table 8). From all available data the average binding efficiency of mutant RNA C_4 was 3.2 (range 1.5 - 5.6) times that of wild type RNA, while the corresponding values for the mutant RNA C_3/C_4 RNA were 0.31 (0.14 - 0.4). In one instance only, a low level of binding of mutant C_3 RNA was observed (cf. Table 8, expt. 5).

The fact that the mutant RNA C_3/C_4 was able to stably bind a ribosome at the coat cistron initiation site despite the absence of an A-U-G triplet raised the question as to whether this binding was dependent on fMet-tRNA. Preliminary experiments showed that low salt washed ribosomes did not show a strong dependence on fMet-RNA for binding to wild type RNA; in the absence of fMet-tRNA 70 S complex formation was about 19% of that found in its presence, as reported previously by Steitz (1969). However, using high salt washed ribosomes prepared according to van Duin & van Knippenberg (1974), 70 S complex formation in the absence of fMet-tRNA was less than about 0.3% for both wild type and mutant RNA, and 12.0% and 17.1%, respec-

TABLE 8

Summary of the analysis of the relative binding efficiency of mutant and wild type RNA to ribosomes

Expt.	Preparation used as template*	[α-^{32}P]NTP used for labeling	Distribution of RNA species in Qβ replicase-RNA complex** wt	: C_3	: C_4	: C_3/C_4	Distribution of RNA species in 70 S ribosome-RNA complex (relative binding efficiency)+ wt	: C_3	: C_4	: C_3/C_4
1‡	A	ATP	1	1	1.3	2.9‡	1 (1	< 0.1 < 0.1	1.9 1.5	0.4 0.14)+
2	B	ATP	1	2	1	5	1 (1	< 0.1 < 0.1	5.6 5.6	2 0.4)+
3	C	ATP	1	2.5	2.5	5	1 (1	< 0.1 < 0.1	6 2.4	2 0.4)+
4	C	UTP		n.d.			(((	1 1	))	0.1° 0.12)+
5	D	CTP		n.d.			1	0.1	1.1	0.1

‡ The analysis of experiment 1 is described in detail in the Results section. WT, wild type; C_3, C_4 and C_3/C_4 are the mutant RNAs described in Fig. 13.

* The following scheme indicates the origin of the templates used. The values in parantheses give the amount of RNA used to initiate in vitro replication, the value without parantheses gives the yield of 30 S RNA after purification.

minus strand preparation 4A ($\overline{\text{HOCMP}}$) No. 1 (0.4 µg)→ 6.4 µg (0.88 µg)→ 36 µg (2 µg)→ 15 µg (A)

minus strand preparation 4A ($\overline{\text{HOCMP}}$) No. 2 (0.1 µg)→ 1.1 µg (0.25 µg)→ 9.6 µg (1 µg)→ 5.2 µg

→ 20 µg ↗ 15 µg (D)

→ 20 µg ↘ (5 µg) → 12 µg ↗ (5 µg)→ 50 µg (B)

→ 20 µg ↘ (5 µg) → 12 µg ↘ (5 µg)→ 76 µg (C)

** Taken to indicate the ratio of RNA species in The RNA preparation, cf. Results section.

+ Calculated as the ratio $\frac{\text{proportion in ribosome complex}}{\text{proportion in replicase complex}}$

° The analysis of [^{32}P]UMP-labeled RNA cannot distinguish between wt, C_3 and C_4 The distribution in the RNA preparation is assumed to be the same as in experiment 3 where the same template was used, i.e. (wt + C_3 + C_4) : C_3/C_4 = 1 : 0.83.

-74-

tively, in the presence of fMet-tRNA (Fig. 20). The mutant RNA preparation used in this experiment contained 15% of the C_3/C_4 RNA. If the binding efficiency of this mutant RNA species in the absence of fMet-tRNA were not less than 0.14 that of the wild type (in the presence of fMet-tRNA, cf. Table 8), then 17.1 x 0.14 x 0.15 = 0.36% of the mutant RNA preparation should have been found in the 70 S complex (above the background value of 0.3%), which was not the case. There is thus no support for the idea that C_3/C_4 mutant RNA is bound by ribosomes in the absence of fMet-tRNA; it must however be considered that the sensitivity of our assay was borderline.

Fig. 20 Binding of ribosomes to wild type and mutant Qβ RNA in the presence or absence of fMet-tRNA. High salt-washed ribosomes and crude initiation factors (prepared as described by Van Duin & Van Knippenberg, 1974) were the gift of Dr. G. van Dieijen, University of Leiden. The binding mixture contained (in 200 µl) 60 mM-Tris-HCl (p H 7.5), 120 mM-NH_4Cl, 8 mM-$MgCl_2$, 0.6 mM-dithriothreitol, 0.5 mM-GTP, 2.4 OD_{260} units of 30 S ribosome, 3.0 OD_{260} units of 50 S ribosome and a saturating amount of crude initiation factors. When required, 165 µg of unfractionated fMet-tRNA were added. After 5 min at 37 ^{o}C, about 85 µg of [^{32}P]AMP-labeled (a) wild type or (b) mutant plus strands were added and incubation was carried out for another 10 min at 37 ^{o}C. The mixture was layered on 10 - 30 % sucrose gradients and analyzed as described in Materials and Methods.
●——● ; in the absence of fMet-tRNA (specific activity of wild type plus strands, 8.0 x 10^5 cts/min/µg, of mutant plus strands, 2.2 x 10^6 cts/min/µg). o——o; in the presence of fMet-tRNA (specific activity of wild type plus strands, 2.0 x 10^7 cts/min/µg, of mutant plus strands, 5.4 x 10^7 cts/min/ g). Inset: The 70 S region shown in expanded scale.

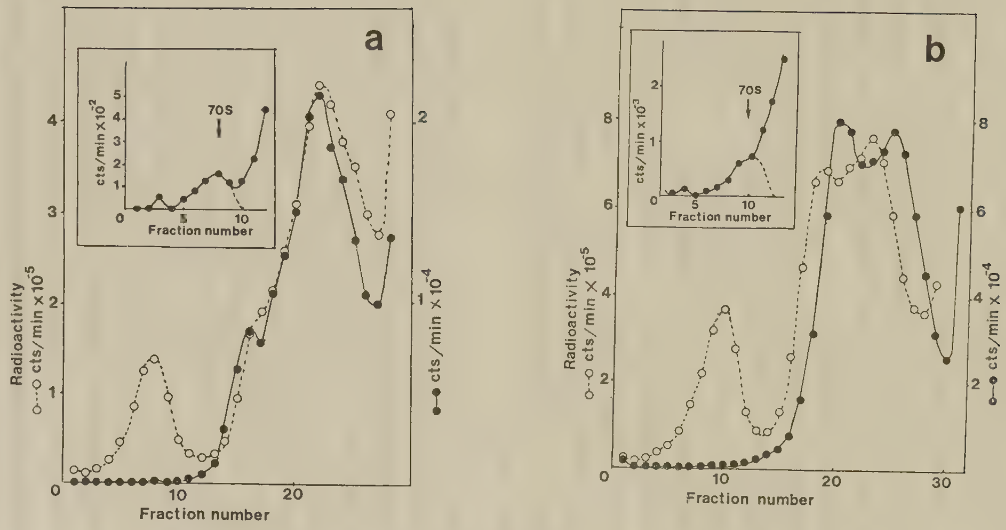

a
Radioactivity
cts/min x 10^-5
cts/min x 10^-4
Fraction number
70S
cts/min x 10^-2
Fraction number
b
Radioactivity
cts/min X 10^-5
cts/min X 10^-4
Fraction number
70S
cts/min x 10^-3
Fraction number

Fig.20

DISCUSSION

(A) Interaction of ribosomes with Qβ RNA in the presence and in the absence of fMet-tRNA

In order to study the molecular basis of mRNA-ribosome interactions *in vitro*, we have isolated the segment of Qβ RNA bound by 70 S or 30 S ribosomes in the presence or in the absence of initiator fMet-tRNA. There has been a certain discrepancy among different reports in respect to the requirement of fMet-tRNA for ribosome binding to the mRNA. Several workers demonstrated that, even in the absence of fMet-tRNA, 30 S ribosomes are still capable of binding to mRNA whereas 50 S ribosomes are not (Greenshpan & Revel, 1969; Vermeer et al., 1973; Szer & Leffler, 1974), whereas Noll and Noll (1974) as well as Jay & Kaempfer (1975) argued in favor of an indispensable role of fMet-tRNA for ribosome binding.

We have shown in this paper that the Qβ RNA segments recovered from binding complexes formed in the presence or absence of fMet-tRNA fall into two sets. One set was derived from regions comprising protein synthesis initiation sites (mainly from the coat and to a lesser degree from the replicase cistron), and the other from non-initiator regions. The major non-initiator fragments were P-2a, derived from the A-1 cistron (A. Shapira & M.A. Billeter, in press), P-4 from the end of the A-2 cistron (H. Walliser, personal communication), and P-15 from an as yet unidentified region. The amount of initiator region found in the 70 S complex was increased twentyfold by addition of fMet-tRNA while the amount of non-initiator fragments was unchanged; the 30 S complex consisted exclusively of noninitiator fragments, the amount

of which was decreased threefold when fMet-tRNA was added. The 30 S complexes formed with unfractionated ribosomes in the presence of fMet-tRNA, or with purified 30 S subunits under initiation conditions (unpublished results) yielded no significant amounts of initiator fragments under our assay conditions. Although it is possible that the 50 S ribosome may play a role in the correct positioning of the 30 S ribosome on Qβ RNA in the presence of fMet-tRNA, it seems more likely that our failure to find significant amounts of initiator fragments in the 30 S complex is due to the lack of protection of the bound sequences in the absence of the 50 S subunit. However, Legon et al. (1977), using unfractionated E.coli ribosomes and fl mRNA, have reached the conclusion that the same RNA regions are protected in the 30 S as in the 70 S complex formed in the presence of fMet-tRNA. We do not know whether the difference in these results is due to the different mRNAs used or to some difference in experimental technique.

It must be noted that studies on the ribosome binding to mRNA involve a multi-step assay which includes RNAase treatment, zonal centrifugation and binding to Millipore, so that the results depend on several parameters, such as the resistance of the bound RNA to RNAase, the dissociation constant of the complex, in particular at elevated hydrostatic pressure (Infante et al., 1971). It is possible that unstable complexes, even if they are of functional importance, may escape notice; on the other hand, even a stable complex need not be functionally essential in the initiation process.

Nucleotide sequence analysis of the non-initiator fragment revealed that all three fragments show extensive complementarity to the 3' terminal region of 16 S rRNA. Oligonucleotide P-2a, which has a free energy of helix formation of -16.9 kcal/

mol with the 3' end of 16 S RNA, was shown to attach to the 3' end of rRNA when it was incubated with ribosomes. The results strongly suggest that the specific interaction between ribosome and Qβ RNA in the absence of fMet-tRNA can be attributed mainly to RNA-RNA interaction.

The possiblility that the 3' terminal region of 16 S rRNA (which is part of the 30 S ribosome) could interact directly with mRNA has been pointed out by Shine & Dalgarno, who noticed that all procaryotic mRNA initiation sites known at the time were preceded by a sequence complementary to the 3' terminal region of ribosomal 16 S RNA, and suggested that an interaction between the two served to direct the ribosome to the correct initiator triplet (Shine & Dalgarno, 1974). In fact, a recent survey (Steitz et al., 1977) shows that all mRNAs, except λ Prm mRNA which starts with the initiator pppA-U-G (Walz et al., 1976), possess the sequence complementary to the 16 S rRNA terminal region in their leader region; however it should be noted that the extent of complementarity varies from mRNA to mRNA and this does not necessarily correlate with the efficiency of ribosome binding. For example, in the case of Qβ RNA, the ribosome binds exclusively to the coat cistron where possible base paring of the "Shine-Dalgarno" region with 3' end of 16 S rRNA is limited to 4 nucleotides including one G-U pair ($\Delta G = -7.9$ kcal/mol), whereas the efficiency of ribosome binding to the replicase cistron is very low even if the "Shine-Dalgarno" region comprises up to 6 nucleotides ($\Delta G = -10.1$ kcal/mol) (cf. Table 1 & Fig. 7). It is obvious that other factors are involved in the process of ribosome binding, as discussed below.

Steitz & Jakes (1975), Steitz & Steege (1977) and we in this paper have demonstrated that the 3' terminal segment of 16 S rRNA in intact ribosomes is capable of binding a complementary

sequence. A few comments are to be made in comparing our work with that of Steitz and her colleagues. These works, in order to demonstrate the occurence of the expected RNA-RNA hybrid, made use of two purified RNA fragments derived from ribosome binding sites with the most extensive complementarity to the 3' terminal region of 16 S rRNA (i.e. R-17 A site & λ Pr site; Steitz et al., 1977b), whereas we have used intact Qβ RNA and isolated the sequence which was bound to the ribosomes. Second, as shown in this paper, at least in the case of Qβ RNA, ribosomes, in the absence of fMet-tRNA, do not bind to the Shine-Dalgarno regions preceding the initiation triplet, but to sequences which are far from any initiation sites. Our results, therefore, not only demonstrate that the 3' terminal region of 16 S rRNA provides the driving force for the binding of 30 S ribosomes to mRNA in the absence of fMet-tRNA but, at the same time that this RNA-RNA interaction is insufficient for the correct placement of the ribosome in the case of Qβ RNA. On the other hand, the absolute requirement of fMet-tRNA for ribosome binding to the initiation site implies the importance of codon-anticodon interaction and/or a conformational change of the 30 S ribosome in the presence of fMet-tRNA for this process.

It is of interest in this connection that when Steitz (1973a) incubated B.stearothermophilus ribosomes with Qβ RNA (with or without fMet-tRNA) at 65 $^{\circ}$C and treated the complex with RNAase A, two fragments were retained on the 70 S ribosomes. One of these, the so-called non-initiator fragment b, was identical with P-4; the other, an oligonucleotide designated "non-initiator fragment a" (NIFa), with the sequence A-A-A-C-U-G-A-A-A-G-G-G-G-A-G-A-U-U-A-C-A-C-U-C-G-A-A-C-G-C-C-G-U-G-A-U-C-G was never observed by us to bind to E.coli ribosome. Both fragments are complementary to the 3' termi-

nal regions of the 16 S ribosomal RNA from both E.coli and B.stearothermophilus (Sprague et al., 1977) (Fig. 7). The calculated free energies of complex formation between P-4 or NIFa on the one hand and B.stearothermophilus 16 S RNA on the other, -12.7 kcal/mol in both cases, were greater than the free energies for complex formation between these oligonucleotides and E.coli 16 S RNA, namely -11.1 and -9.5 kcal/mol, respectively. The difference in the free energies of the interactions of P-4 and NIFb with E.coli 16 S RNA seem too small to explain the fact that P-4(NIFb) but not NIFa was bound. Even more striking are the findings that (1) P-2a, which has a free energy of helix formation of -16.4 kcal/mol with B.stearothermophilus 16 S rRNA, was not bound to B.stearothermophilus ribosomes under condition where P-4 and NIFa were, although the corresponding ΔG values are lower, and that (2) P-4 and NIFa were bound to B.stearothermophilus ribosomes at 65 ^{o}C and virtually not at 49 ^{o}C. To explain the latter observation, Sprague et al. (1977) have suggested that P-4 and NIFa are masked by secondary structure at the lower and exposed at the higher temperature. Inasmuch as it is permissible to compare results obtained in different laboratories by slightly different methods, the findings that E.coli ribosomes bind to the P-4 region even at 37 ^{o}C could be taken to mean that certain secondary structures can be melted out at low temperature by E.coli ribosomes, but not by B.stearothermophilus ribosomes. It has in fact been shown that translation of heteropolymeric RNA by B.stearothermophilus ribosomes is more sensitive to the secondary structure of the template RNA than is translation by E.coli ribosomes (Chen & Nakamoto, 1978). This could be attributed to the fact that the ribosomal protein S1 of E.coli has the capacity of melting out RNA (Bear et al., 1976; Szer et al., 1976) and that B.stearothermophilus lacks a protein similar to S1 (Isono & Isono, 1976). The function of this protein will be discussed later.

(B) Importance of 16 S rRNA 3' terminal region in the formation of 70 S initiation complexes between ribosomes and Qβ RNA

The most critical question regarding the hypothesis of Shine and Dalgarno (1974) is whether the postulated interaction plays a role in ribosome binding, and if so, to what extent. In particular, our finding that the nonanucleotide P-2a is by itself capable of binding to the 30 S ribosome convincingly shows that an RNA segment with sufficient complementarity to the 3' terminal region of 16 S rRNA can bind to the 30 S ribosomes no mater whether the RNA fragment originates from initiation site (R-17 A_1 site; Steitz & Jakes, 1975. λ Pr site; Steitz & Steege, 1977) or not (non-initiator site P-2a, P-15 and P-4 in this paper; possibly NIFa as well as NIFb; Steitz, 1973a). The fact that the synthetic polynucleotide A-U-G-U-U-U... serves as mRNA under conditions where initiation factors are required for protein synthesis (Thach et al., 1966) as well as the existence of λ Prm mRNA which does not contain any leader sequence and yet serves as messenger for repressor protein synthesis (Walz et al., 1976) may be taken as an argument against the general requirement of RNA-RNA interactions of the Shine-Dalgarno type during the initiation of protein synthesis. On the other hand, Dunn et al. reported that 0.3 mRNA of phage T7, containing a point mutation which diminishes the extent of Shine-Dalgarno interaction, is no longer bound to ribosomes at the original initiation site but at a region further downstream which contains an A-U-G triplet (but no Shine-Dalgarno region). Moreover, they found that a suppressor mutation which generates a more stable Shine-Dalgarno interaction restores ribosome binding to the original site. These results strongly suggested the importance of Shine-Dalgarno interaction (Dunn et al., 1978).

We have taken a different approach to assess the role of the 3' terminal sequence of 16 S rRNA in the process of 70 S initiation complex formation. Our experiments demonstrate that the nucleotide P-2a, which binds stably to the 3' terminal region of 16 S ribosomal RNA (ΔG = -16.9 kcal/mol), strongly inhibits fMet-tRNA dependent 70 S complex formation with Qβ RNA. Since the formation of complexes between pA-U-G and the 70 S ribosome is not inhibited by the oligonucleotide, we conclude that inhibition (or destabilization) of Qβ RNA binding is not due to an effect on the interaction between fMet-tRNA and the initiation triplet or on the association of the 30 S and 50 S ribosome subunits. The results presented here show that the interaction between the 3' terminal region of 16 S rRNA and the coat cistron of Qβ RNA is in fact critical for efficient and stable initiation complex formation.

If P-2a blocks the formation of Shine-Dalgarno (or other RNA··RNA) interactions, then ribosomes might attach to Qβ RNA only by virtue of the fMet-tRNA-AUG interaction. If we assume that there are no other important interactions between ribosomes and natural mRNA, the rate of formation or stability of complexes between 70 S ribosomes on the one hand and the triplet A-U-G or any mRNA on the other should become similar in the presence of oligonucleotide P-2a. We have not studied the kinetics of the inhibition and it is possible that upon prolonged incubation of P-2a-blocked ribosomes with Qβ RNA binding might increase with time, either because of an exchange reaction between Qβ RNA and P-2a or because the codon-anticodon interaction by itself might suffice for a slow rate of complex formation.

Which region of the Qβ RNA is then recognized by the 3' terminal sequence of 16 S rRNA for initiation complex forma-

tion? At present, we do not know the answer. In view of the Shine-Dalgarno hypothesis (Shine & Dalgarno, 1974), as well as of the findings of Dunn et al, (1978), it is likely that such an interaction occurs at the region preceding the A-U-G codon of the coat cistron. However, we would like to point out that the coat cistron possesses a conjectured Shine-Dalgarno sequence capable of forming only 3 Watson-Crick plus one G-U base pairs with 3' terminal region of 16 S rRNA ($\Delta G = -7.9$ kcal/mol), whereas all non-initiator regions bound by ribosomes can form far more stable interactions ($\Delta G = -11.1 \sim -16.9$ kcal/mol: cf. Fig. 7). Thus, the contribution due to the interaction of the initiator tRNA may override the difference in affinity between the Qβ RNA sites complementary to the ribosomal RNA. We should also consider the possibility that the ribosome non-initiator complex serves as a precursor which is then rearranged to the correct initiation complex following the addition of fMet-tRNA. Jay & Kaempfer (1975) found that the complex formed between ribosomes and R17 RNA in the absence of fMet-tRNA was not resistant to edein, an inhibitor of the attachment of mRNA but not the binding of fMet-tRNA, whereas the complex formed in the presence of fMet-tRNA was resistant to the inhibitor. From this result they concluded that the complex formed in the absence of fMet-tRNA was not a precursor of the initiation complex. We think this argument is not convincing, in particular in view of our finding that binding of 30 S ribosomes does not occur to the protein initiation sites of Qβ RNA; this may mean that the edein-sensitive initiator site on the ribosome is not protected.

In view of the conclusion of Gualerzi et al. (1977), that either mRNA or fMet-tRNA may first attach to the ribosome, it is possible that the binding of ribosomes to non-initiator regions yields a 30 S precursor complex, which on addition of fMet-tRNA rearranges to the stable initiation complex.

(C) Role of initiator A-U-G in the 70 S initiation complex formation

The results discussed above demonstrated the importance of fMet-tRNA as well as of the 3' terminal region of 16 S rRNA in the correct placement of the ribosome on the mRNA. In view of the importance of fMet-tRNA, the question arose as to whether the initiation triplet itself is an essential signal for the ribosome binding. In order to answer the question, we have synthesized a Qβ RNA preparation containing RNA species with G → A transitions in either the third (C_3), the fourth (C_4) or in the third and fourth (C_3/C_4) positions of the coat cistron. In mutants C_3 as well as C_3/C_4 the A-U-G sequence was thus replaced by A-U-A.

Binding experiments carried out in the presence of fMet-tRNA, with an excess of low salt-washed ribosomes, showed that mutant C_3RNA, in which the initiator A-U-G was replaced by A-U-A, did not form a 70 S complex in three out of four experiments; the limit of detection was 5 to 10 % that of wild-type RNA. In one experiment binding of C_3 RNA was measured at a low level (cf. Table 8). Somewhat unexpectedly, the double mutant with the sequence A-U-A-A-C-A (instead of A-U-G-G-C-A) bound ribosomes with an efficiency 10 to 40% that of wild-type RNA, and mutant C_4 RNA, with the conserved A-U-G triplet, formed a 70 S complex 1.5 to 5.6 times more efficiently than wild-type RNA.

At first sight these findings seem paradoxical: the absence of the A-U-G triplet precludes 70 S complex formation in mutant C_3 RNA while in the double mutant C_3/C_4 it does not. An explanation is, however, suggested by the finding that the ribosome binding capacity of mutant C_4 is increased above normal: namely that an A residue following the initiation

triplet stabilizes the interaction between fMet-tRNA and the ribosome binding site (Fig. 21). Such a stabilization could come about by stacking interactions (Grosjean et al., 1976) or, as shown in Figure 21, by the formation of a fourth basepair with the anticodon loop of fMet-tRNA. Uhlenbeck et al. (1970) have in fact shown that the molar association constant (in the absence of ribosomes) of fMet-tRNA with A-U-G-A is more than ten times geater than that with A-U-G, A-U-G-G, A-U-G-C or A-U-G-U.

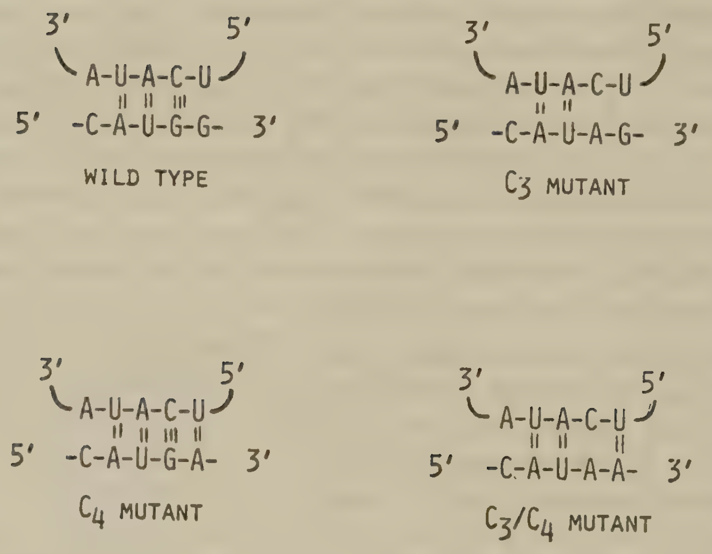
While the ribosome binding site of mutant C_3 RNA can hydrogenbond to only two nucleotides of the anticodon, that of mutant C_3/C_4 RNA can again form three base-pairs, although in this case the additional pair is isolated from the other two. It is clear from the calculations of Gralla & Crothers (1973) and Tinoco et al. (1973) that such an interaction would provide little stability if it took place in solution, and we must postulate a special effect in the case of codon-anticodon interactions (Grosjean et al., 1976).

The results discussed above, therefore, document the essential contribution of the interaction between "ribosome binding site" and fMet-tRNA for initiation complex formation. This is in perfect agreement with the results showing that correct placement of the ribosome requires fMet-tRNA, at least in the case of Qβ RNA. In the light of these findings, we will discuss further, elements involved in ribosome binding.

(D) RNA-RNA interactions in ribosome binding process

Our results show that two kinds of RNA-RNA interaction play a role in the formation of correct, efficient and stable 70 S initiation complexes by E.coli ribosome: (1) the inter-

Fig. 21 Possible Watson-Crick base pairing between the initiation regions of mutant Qβ RNAs and the anticodon loop of fMet-tRNA.
The sequence of the fMet-tRNA is from Dube et al. (1968). Details are discussed in the text.



action between the ribosome binding site (A-U-G region) and the anticodon region of fMet-tRNA (2) the interaction between the 3' terminal region of 16 S rRNA and mRNA (probably at the Shine-Dalgarno region). It seems obvious from the case of λ Prm mRNA, which starts with the initiator sequence pppA-U-G... (Walz et al., 1976) as well as from translation experiments carried out with synthetic polynucleotide such as A-U-G-U-U-U... (Thach et al., 1966) that the fMet-tRNA A-U-G interaction can suffice for ribosome binding. However, in the case of the mRNA for repressor synthesis, the efficiency of translation of the repressor gene via Prm promotor is thought to be an order of magnitude less than that of Pre, the same mRNA preceded by a leader sequence comprising a Shine-Dalgarno region (Ptashne et al., 1976). Accordingly, as we have shown in this paper, the binding of the trinucleotide pA-U-G to ribosomes appears to be far weaker than the binding of Qβ RNA, as judged by the molar concentration of the RNA required for efficient ribosome binding. On the other hand, the fact that the formation of 70 S complex between wild type RNA and ribosomes is strongly inhibited by P-2a whereas pA-U-G binding is not, demonstrates that additional interactions of Qβ RNA with the ribosome also play a role. It seems that the importance of the two types of RNA-RNA interaction depends on the structure of the mRNA, in particular at the ribosome binding site and therefore one or the other (or both) plays a major role depending on the species of mRNA; maximal expression of the gene probably requires both interactions (Steitz & Jakes, 1975, Taniguchi & Weissmann, 1978). Thus, in the case of R17 A site, where the Shine-Dalgarno interaction comprises 7 base pairs, fMet-tRNA may play a minor role in ribosome binding (Steitz, 1973b), whereas λPrm mRNA, which lacks a leader sequence, may show an absolute requirement for fMet-tRNA. In the case of Qβ RNA,

both interactions appear to be critical for efficient and stable binding of ribosome. In this connection, it is interesting to note that when phage T_4 carries an A-U-G → A-U-A substitution in the initiation codon of the rIIB mRNA, it became temperature sensitive and that, at the permissive temperatrure (i.e. 25 ^{o}C), wild type rIIB protein is synthesized (D. Belin, personal communication). As might be predicted from what we discussed above, this mRNA in fact possesses polypurine tracts in the leader region which are potentially capable of hydrogen bonding to the 3' terminal region of 16 S rRNA.

(E) Role of secondary (or tertiary) structure of mRNA in the ribosome binding process

It is clear that the RNA-RNA interactions discussed above do not explain all secrets of ribosome binding. For example, a scan of nucleotide sequences of phage MS2 RNA reveals a number of A-U-G or G-U-G triplets both within and without the correct reading frame, which are preceded by a potential Shine-Dalgarno sequence but appear never to be bound to ribosomes (Fiers et al., 1976). It has been proposed that the secondary structure of the RNA functions as a negative effector, occluding either the initiator triplet or the polypurine sequence. Lodish has first shown that f2 RNA treated with formaldehyde, which irreversibly denatures RNA, directs the synthesis of several new peptides (Lodish, 1970b). It is obvious that even true initiation sites are masked by secondary (or tertiary) structure since the replicase initiation site is less than 10% as effective in ribosome binding as the coat cistron site (Table 1), and no binding was observed at the A_2 protein site. Since the situation is similar in many

phage RNAs studied, the experession of those genes is generally believed to be accomplished either following the alteration of the secondary structure by translating ribosomes (Lodish, 1975) or when the RNA is till in nascent state and cannot yet form its native conformation (Staples et al., 1971). In vitro it has also been shown that, in addition to formaldehyde treatment, fragmentation (Steitz, 1972) or heating of phage RNA (Fukami & Imahori, 1971) increase ribosome binding at the phage replicase and A protein cistrons. On the other hand, certain features of secondary or tertiary structure might affect protein synthesis positively. It has been proposed that in the case of codon anticodon interactions stacking interactions similar to those proposed for tRNA-tRNA association (Grosjean et al., 1976) may play a role and that the A-U-G triplet must be not only free of secondary structure but also supported by an arm of double helical structure as in the case of the anticodon loop of tRNA (Grosjean et al., 1978). Although at present there is no experimental evidence supporting this view, it might be significant that many (but not all) initiation triplets, including that of the Qβ coat cistron, are located on a RNA loop (Steitz, 1975).

It is possible that the interaction of ribosomes by virtue of the 3' end of 16 S rRNA occurs not in the linear vicinity of the initiator triplet but very close to it due to the three dimensional structure of the RNA, so that a possible fMet-tRNA-induced relocation of the ribosome may occur without substantial movement. If this is the case, the conformation of the RNA may be very critical. In this connection it is of interest to note that a supressor mutation of T7 0.3 mutant mRNA (A-U-G → A-C-G) is located in the region proximal to the 3' end, which is very far from the original ribosome binding site (Dunn et al., 1978).

(F) Involvement of proteins in the ribosome binding process

The two types of RNA-RNA interaction described above are important elements contributing to the specific binding of ribosomes to the initiation site of mRNA. On the other hand, much evidence has accumulated documenting the role of proteins like S1 (van Duin & van Knippenberg, 1974; van Dieijen et al., 1976; Dahlberg, 1974; Szer & Leffler, 1974; Hermoso & Szer, 1974), S-12 (Held et al., 1974; Goldberg & Steitz, 1974) and initiation factors, in particular initiation factor 3 (1F3) (Iwasaki et al., 1968) in the initiation of protein synthesis. Yet, the mechanism of their action remains totally unclear, in part because we know even less about the molecular basis of RNA-protein (or protein-protein) interactions than about that of RNA-RNA (or in general, nucleic acid) interactions. Ribosomal protein S1 is important not only for phage RNA ribosome binding and translation, but also for translation of host mRNAs (van Dieijen et al., 1977) as well as of poly U (van Duin & van Knippenberg, 1974, Yokota et al., 1977). The precise mechanism of action of this protein is unclear, however, several properties are reported, (1) S1 is present in stoichiometric amount in E.coli cells relative to other ribosomal proteins (van Knippenberg et al., 1974), (2) S1 binds strongly to single-stranded RNA, in particular to pyrimidin-rich RNA like poly U (Bear et al., 1976; Draper & von Hippel, 1976), (3) S1 has a function in disrupting the ordered state of RNA (RNA unwinding) (Bear et al., 1976; Draper & von Hippel, 1976; Szer et al., 1976). (4) S1 is identical to the α-subunit of Qβ replicase (Inoue et al., 1974; Wahba et al., 1974). Since chemical cross-linking (Kenner, 1973; Czernilofsky et al., 1975) and binding studies (Dahlberg & Dahlberg, 1975) locate

S1 adjacent to the 3' terminus of 16 S rRNA on the ribosome, it has been suggested that S1 interacts with this region of RNA and accelerates Shine-Dalgarno interactions by disrupting the secondary structure of 16 S RNA or stabilizes the RNA-RNA interaction (Dahlberg & Dahlberg, 1975; Steitz et al., 1977). However, NMR studies on the 49 nucleotide long RNA fragment from the 3' end of 16 S RNA provided no evidence for such disruption of secondary structure (Baan et al., 1977). Moreover, a recent study of 30 S ribosomes lacking the 3' terminal segment of 16 S rRNA demonstrated that the ribosome is still capable of binding S1 (Laughrea & Moore, 1978). On the other hand, van Dieijen et al. (1976) reported that when MS2 RNA is pre-treated with formaldehyde, the ribosome no longer needs S1 for its binding to the RNA. The authors claim that the function of S1 is to disrupt the three dimensional structure of mRNA allowing the ribosome to bind. It is, however, not clear whether ribosomes are bound to the correct initiation site or not when formaldehyde-treated RNA is used, particularly in view of Lodish's finding that indiscriminate chain initiation occurs when f2 RNA is treated with same reagent (Lodish, 1970b). Obviously, S1 is essential for ribosome binding to Qβ RNA as well. We do not know yet whether S1 is required for ribosome binding to noninitiator sites nor do we understand the mechanism of S1 function in the binding of ribosomes to Qβ RNA; possibly there are numerous possibilities as to how this protein may affect the translational system. It should be noted that a counterpart of S1 in B. stearothermophilus seems to be absent (Isono & Isono, 1976). As far as initiation factors are concerned, the general properties of 1F-2 in the binding of initiator tRNA to the ribosome (Miller & Wahba, 1973) as well as of 1F-1 in catalytic recycling of 1F-2 (Benne et al., 1972) are well documented. The function of 1F-3 is (1) to promote

the binding of natural mRNA to ribosomes (Iwasaki et al., 1968), (2) to shift the equilibrium of ribosomes and their subunits towards dissociation by binding to the small subunits (Subramanian et al., 1969). How lF-3 stimulates ribosomes binding to natural mRNA is still not clear; a recent study of Steitz et al. (1977a) shows that by adding lF-3 as well lF-2 and Sl to the incubation mixture, one gets a different extent of stimulation of ribosome binding to the three cistrons on R17 RNA. The authors claim that the functions of those proteins are to facilitate or stabilize Shine-Dalgarno interactions so that ribosome binding to the cistron having a short Shine-Dalgarno sequence is more stimulated by those proteins. There is evidence showing that lF-3 and the 3' terminus of 16 S rRNA are related topographically on the ribosome (van Duin et al., 1975).

Apart from Sl and the initiation factors, protein S12 has been reported to play a role in the initiation of protein synthesis (Held et al., 1974; Goldberg & Steitz, 1974). Although the possibility of S-12 interacting with mRNA cannot be ruled out, it seems likely that the function of this protein lies in keeping the ribosome in an active conformation. The evidence showing that S 12 is cross-linked to lF-3 and lF-2 (Hawley et al., 1974; Bollen et al., 1975) implies that S 12 may mediate factor function, indirectly affecting ribosome recognition of initiator regions; it is however also possible that S 12 affects comformation of the 3' terminal region of the 16 S rRNA.

SUMMARY

E.coli ribosomes and Qβ[^{32}P] RNA were incubated with or without fMet-tRNA under protein synthesis initiation conditions, treated with RNAase A, and centrifuged through a sucrose density gradient. The sample incubated with fMet-tRNA gave a main radioactivity peak in the 70 S region, which consisted predominantly of coat cistron initiator fragments. After incubation without fMet-tRNA, equal amounts of radioactivity were found in the 70 S and the 30 S regions, but in both peaks almost all of the radioactivity was due to three RNAase A-resistant oligonucleotides, A-G-A-G-G-A-G-G-Up (P-2a), A-G-G-G-G-G-Up (P-15), G-G-A-A-G-G-A-G-Cp (P-4). These three oligonucleotides are derived from 3 different RNA regions, none of which is close to a protein initiation site. All three fragments show striking complementarity to the 3' terminal region of E.coli 16 S RNA. Ribosomes incubated with an RNAase A digest of Qβ[^{32}P]RNA bound almost exclusively oligonucleotide P-2a; treatment with cloacin DF13 cleaved off a complex consisting of the 49 nucleotide-long 3' terminal fragment of 16 S RNA and oligonucleotide P-2a. These experiments show that the interaction of 30 S ribosomes with the region preceding the initiator codon of the Qβ coat cistron (Shine-Dalgarno interaction) is insufficient to direct correct placement of the ribosome on the Qβ RNA and that an additional contribution from fMet-tRNA, possibly the interaction with the A-U-G codon is required for ribosome binding to the initiator region.

In order to test the functional importance of the RNA-RNA interaction proposed by Shine and Dalgarno, the 3' terminal region of 16 S RNA in the E.coli ribosome was first blocked

by oligonucleotide P-2a and the capacity of those ribosomes to form 70S initiation complexes with Qβ [^{3}H]RNA was studied. At a P-2a to ribosome ratio of 1, about 60 % inhibition of 70 S complex formation was observed and at a ratio of 4, inhibition was 90 %. RNAase T_1 treatment of P-2a completely abolished the inhibitory activity of P-2a; fMet-tRNA-dependent 70 S ribosome binding to ^{32}P-labeled pA-U-G was not inhibited at all by the oligonucleotide.

Finally, Qβ RNAs bearing mutations at the initiator region of the coat cistron were generated <u>in vitro</u> to assess the role of A-U-G initiator codon in the process of initiation complex formation with E.coli ribosomes. Qβ plus strands with a 70 S ribosome bound to the coat cistron initiation site were used as templates for Qβ replicase. When the replicase reached the ribosome, RNA synthesis stopped. The ribosome was removed and elongation was continued in a substrate controlled, stepwise fashion. The nucleotide analog N^4-hydroxyCMP was introduced into the positions complementary to the third and fourth nucleotides of the coat cistron. The minus strands were elongated to completion, purified and used as template for Qβ replicase. The final plus strand preparation consisted of four species, with the sequence -A-U-G-G- (wild type), -A-U-A-G- (mutant C_3), -A-U-G-A- (mutant C_4) and -A-U-A-A- (mutant $C_{3/4}$) at the coat initiation site. The ribosome binding capacity of the mutant RNAs relative to wild type was < 0.1 (C_3), 3.2 (C_4) and 0.3 ($C_{3/4}$). The finding that mutant C_3 no longer formed an initiation complex suggests that the interaction of the ribosome binding site with fMet-tRNA plays an essential role in the formation of the 70 S initiation complex. The fact that mutant C_4 bound more efficiently than wild type and that mutant $C_{3/4}$ showed substantial ribosome binding capacity whereas the single mutant C_3 did not, can be explained by assuming that an A residue

following the A-U-G triplet interacts with a complementary U residue in the anticodon sequence. In the case of $C_{3/4}$ this additional base pair may offset the reduced codon-anticodon interaction resulting from the modification of the A-U-G codon.

The results presented here thus lead to the following conclusion: Two kinds of RNA-RNA interaction, that of Qβ RNA with the 3' terminus region of 16 S rRNA on the one hand and with the anticodon loop of fMet-tRNA on the other play an essential role in the process of correct, efficient and stable 70 S initiation complex formation between Qβ RNA and E.coli ribosomes.

MATERIALS AND METHODS

(A) Materials

The following materials were obtained or purified by published procedures: Qβ replicase and host factor I (Kamen et al., 1972), labeled and unlabeled Qβ RNA (Weissmann et al., 1968), Qβ RNA fragments of about 11 S (Pollet et al., 1967), yeast RNA to be used as carrier (Flavell et al., 1974), ribosomes from Escherichia coli Q13 and fMet-tRNA (Kolakofsky & Weissmann, 1973), repurified ribonucleoside triphosophates as well as N^4-hydroxyCTP (Flavell et al., 1974).
Cloacin DF-13 was a gift from Dr. de Graaf, Free University, Amsterdam.

(B) Methods

a) Isolation of ribosome binding sites (for Section I).
The procedure is essentially as described by Steitz (1969). The reaction mixture (final volume, 200 µl) contained: 60 mM Tris-HCl (pH 7.5), 0.12 M NH_4Cl, 6 mM $MgCl_2$, 0.6 mM dithiothreitol, 0.6 mM GTP, 160 pmol of ^{32}P-labeled Qβ RNA (6.3 x 10^5 cts/min/pmol) and 300 pmol of ribosomes (preincubated for 5 min at 37 °C). Unfractionated tRNA, charged with methionine and formylated, was added at 450 pmol when required. After 15 min at 37 °C, the mixture was treated with 2 µg RNAase A for 10 min at 20 °C. The mixture was then layered on a 5 % to 20 % linear sucrose gradient (5 ml) containing 10 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 50 mM NH_4Cl and 5 mM β-mercaptoethanol (buffer B) and centrifuged for 120 min at 40,000 rpm and 4 °C in a SW50 Spinco rotor. Fractions (25) were collected and a 10-µl aliquot was removed from each fraction and mixed with 200 µl of buffer B for determination of absorbance.

The remainder of each fraction was passed through a Millipore filter and washed with about 2 ml of chilled buffer B. The radioactivity retained on the filter was measured by Cerenkov radiation and the ^{32}P-profile determinded. To extract the [^{32}P]RNA the filters were transferred into 15 ml Corex tubes containing 2 ml of buffer (20 mM Tris-HCl (pH 7.5), 2 mM EDTA and 0.5 % SDS) and 2 ml of phenol. The tube was shaken vigorously for about 20 min at room temperature and the aqueous phase, which contained about 90 % of the radioactivity, extracted with phenol once more. After three extractions with an equal volume of ether, the RNA was precipitated by ethanol and dissolved in 10 µl 0.5 mM EDTA. 60 to 90 % of the radioactivity were recovered.

b) Analysis of the ribosome-bound [^{32}P]RNA

The [^{32}P]RNA was denatured in a boiling water bath for 90 sec and analyzed by two-dimensional polyacrylamide gel electrophoresis (De Wachter & Fiers, 1972). After autoradiography, each RNA fragment was extracted from the gel and analysed further either by T_1 fingerprinting or by one-dimensional paper electrophoresis (pH 3.5) after T_1 degradation (Sanger & Brownlee, 1967).

c) Preparation of RNAase A-resistant oligonucleotides of Qβ RNA

^{32}P-labeled Qβ RNA (600 µg or 470 µg, specific activity 4.6 x 10^4 cpm/µg or 7.7 x 10^5 cpm/µg, respectively) was dissolved with 20 µl 20 mM Tris-HCl (pH 7.5) - 2 mM EDTA and digested with 50 µg RNAase A per mg of RNA for 30 min at 37 ^{o}C. The resulting fragments were separated by two-dimensional polyacrylamide gel electrophoresis (De Wachter & Fiers, 1972) and each fragment was eluted from the gel by overnight extraction with 1 ml TNE buffer (50 mM

Tris-HCl (pH 7.5), 100 mM NaCl and 5 mM EDTA). The extract was passed through a 0.1 ml DEAE cellulose column (prewashed with 0.2 M triethylammonium carbonate (TAC); after extensive washing with 0.2 M TAC, the fragment was eluted with 2 M TAC and the residue taken to dryness several times. Finally, the RNA was dissolved in H_2O.

d) Partial T_1 analysis of the oligonucleotides

Uniformly ^{32}P-labeled fragments were digested partially by RNAase T_1 (10^{-4} unit for 20 µg of RNA), the resulting oligonucleotides were separated by two-dimensional electrophoresis, eluted and analyzed further by complete T_1 digestion as described by Sanger and Brownlee (1967).

e) Wandering spot analysis

i) Analysis of P-4

Uniformly ^{32}P-labeled P-4 was partially digested by spleen phosphodiesterase and the products analyzed as described by Rensing and Schoenmakers (1973).

ii) Analysis of P-2a and P-2b

For the preparation of 5' terminally ^{32}P-labeled P-2a, the mixture of P-2a and P-2b (80 pmol) was labeled with polynucleotide kinase (7 units) and [$\gamma^{32}P$]ATP (200 pmol; specific activity 1.1 mCi/nmol) in a final volume of 100 µl as described by Maxam and Gilbert (1977). After 60 min at 37 ^{o}C, the product was chromatographed on Sephadex G-25 equilibrated with 0.2 M TAC, the excluded fractions were pooled and taken to dryness. About 20 % of the starting material were recovered as ^{32}P-labeled material. The product was incubated with ribosomes and the 30 S ribosome-bound fragment (P-2a) was isolated by sucrose gradient centrifugation as described above. Both purified ^{32}P-labeled P-2a and the

mixture of P-2a and P-2b were partially digested by snake venom phosphodiesterase and analyzed essentially as described by Rensing and Schoenmakers (1973).

iii) Analysis of P-15

Oligonucleotide P-15 (about 25 pmol) was prepared by two-dimensional polyacrylamide gel electrophoresis, labeled with ^{32}P at the 5' terminus, partially digested by snake venom phosphodiesterase and analyzed as above.

f) Preparation of Qβ minus strands with N^4-hydroxyCMP substitutions at the positions complementary to the third and fourth nucleotides of the coat cistron.

The experimental plan for this synthesis is outlined and illustrated in Fig. 12.

i) Preparation of the 70 S Qβ RNA-ribosome complex

The conditions are as described by Kolakofsky et al. (1973). The binding mixture contained (in 1.5 ml) 60 mM-Tris-HCl (pH 7.5), 120 mM-NH_4Cl, 8 mM-$MgCl_2$, 0.6 mM-dithiothreitol, 0.5 mM GTP, 2.4 % glycerol, 90 OD_{260} units of "low salt washed" ribosomes, 240 μg of unfractionated fMet-tRNA (^{35}S-labeled at 200 cts/min/pmol) and 2.4 mg of Qβ RNA (^{32}P-labeled at 100 cts/min/μg). After 10 min at 37 °C, the mixture was layered on six 10 % to 30 % linear sucrose gradients (5 ml) in buffer B, and centrifuged for 70 min at 60,000 rev./min (4 °C) in SW65 Spinco rotors. Fractions (25) were collected, the ^{32}P-profile was determined by measuring Cerenkov radiation and the fractions corresponding to the 70 S peak were pooled (about 4 ml). The preparation contained about 270 μg (180 pmol) of [^{32}P] Qβ RNA as ribosome complex.

ii) Synthesis of minus strands up to the position complementary to the 16th nucleotide of the coat cistron (step 1). To 3.8 ml of the 70 S complex preparation

described above the following components were added to give the concentrations indicated (final volume 4.5 ml): Tris-HCl (pH 7.5), 80 mM; $MgCl_2$, 12 mM; GTP, 0.4 mM; ATP 0.2 mM; Qβ replicase, 100 units/ml; host factor I, 100 units/ml. Initiation was allowed to proceed for 5 min at 37 °C and a mixture of [^{3}H]CTP (specific activity, 14,000 cts/min/nmol), UTP and polyethylene sulfonate (Kondo & Weissmann, 1972) was added to give final concentrations of 0.2 mM, 0.2 mM and 0.05 mg/ml, respectively. After 5 min at 37 °C the mixture was chilled and 0.1 vol of 0.5 M-EDTA was added. The preparation was passed through a 1.6 x 45 cm column of Sephadex G-100 equilibrated with 80 mM-Tris-HCl (pH 7.5) at 4 °C. The excluded material was located by the Cerenkov radiation of the [^{32}P]Qβ RNA and the peak fractions, containing 127 pmol of [^{32}P]Qβ RNA and 11 nmol of [^{3}H]CMP were pooled to give a volume of 5 ml. Assuming the chain-length of the minus strand segment synthesized to be about 3000 nucleotides and its average CMP content 25 %, the preparation contained about 15 pmol of minus strands. Thus, template utilization was about 12 %.

iii) Stepwise elongation of the minus strand up to the position complementary to the 5th nucleotide of the coat cistron (step 2)

The replicationg complex described above was incubated at 37 °C for 5 min in the presence of 12 mM-$MgCl_2$, 4 μM each of ATP, CTP and UTP (final volume, 5 ml) in order to elongate the minus strand by 11 nucleotides, up to the point where GTP was required for further synthesis. The complex was separated from unused substrates by chromatography through 1.6 x 40 cm Sephadex G-100 column as above. A pool of 6 ml containing 113 pmol of [^{32}P]Qβ RNA was obtained.

iv) Incorporation of N^4-hydroxyCMP into the positions complementary to the third and fourth nucleotide of the coat cistron (step 3). The preparation described above (6 ml) was adjusted to 50 µg/ml polyethylene sulfonate 12 mM-$MgCl_2$, 8.3 µM-N^4-hydroxyCTP and 2.5 µM-GTP (final volume 6.1 ml). After 5 min at 37 °C, the reaction mixture was divided in two aliquots, one to be used for preparative, the other for analytical purposes.

v) Completion of the minus strand (step 4)
The preparative aliquot (4 ml) was mixed with 4 µmoles of CTP (a 120-fold excess over the N^4-hydroxyCTP), adjusted to 0.25 mM each GTP, UTP and [α-^{32}P]ATP (specific activity 13,000 cts/min/nmol) and incubated at 37 °C for 10 min to complete the minus strands (preparation 4A ($\overline{HOC}$MP)). The analytical aliquot (2 ml) was chromatographed through a Sephadex G-100 column in 80-mM Tris-HCl (pH 7.5). The excluded complex was adjusted to 12 mM-$MgCl_2$ and 1 µM-[α-^{32}P]ATP (specific activity 2.7 x 10^6 cts/min/nmol and incubated for 5 min at 37 °C. Unlabeled ATP (7.5 µmol, a 375-fold excess over the [α-^{32}P]ATP) was added and incubation was continued for 10 min at 37 °C in the presence of CTP, GTP and UTP (0.2 mM each) to complete the minus strand (Preparation 4C, ($\overline{HOC}$MP)). A control preparation, in which CTP was added in the 3rd step instead of N^4-hydroxyCTP, was synthesized in a similar fashion (Preparation 4C (CMP)).

The products were incubated with 0.5 % sodium dodecyl sulfate, 0.5 mg/ml pronase and 25 mM-EDTA for 15 min at 37 °C, and extracted twice with an equal volume of distilled phenol. RNA was precipitated with 2 volumes of ethanol in the presence of 0.1 M-NaCl and collected by centrifugation.

g) Purification and analysis of the minus strands.

The $\overline{HO}CMP$-containing minus stands (preparation 4A ($\overline{HO}CMP$)) were purified free ot template plus strands and ribosomal RNA essentially by the procedure described by Pollet et al. (1967). The RNA was dissolved in 1 ml of 0.5 x SSC and heated for 15 min at 55 °C to convert all minus strands into a doublestranded form by annealing to the excess of plus strand template. Double-stranded RNA was then isolated by chromatography through Sepharose 2B as described by Flavell et al. (1974). Since the minus strand segment labeled by [α-^{32}P]ATP in the last step of the synthesis corresponded to about one fourth of the total length, the amount of minus strands present could be calculated from the relationship:

$$\text{minus strands} = \frac{^{32}\text{P radioactivity}}{\text{spec. radioactivity}} \times \frac{\text{average molecular weight nucleotide}}{\text{molar content of AMP}} \times 4.$$

After Sepharose 2B chromatography 2.3 µg of minus strands were recovered in double-stranded form. After precipitation with ethanol the RNA was dissolved in 0.48 ml of 0.5 mM-EDTA (pH 7.5) containing 70 µg of 11 S Qβ RNA fragments (Pollet et al., 1967). The mixture was heated in a boiling water bath for 90 sec, chilled quickly in an ice bath, and, after addition of 25 µl of 20 x SSC, heated for 15 min at 55 °C. The double-stranded RNA was separated from the plus strand template and the bulk of 11 S Qβ RNA fragments by chromatography on Sepharose 2 B (Flavell et al., 1974).

The RNA, in 0.1 ml 0.5 mM-EDTA (pH 7.5), was denatured in a boiling water bath for 90 seconds. The full-length minus strands were separated from 11 S RNA by centrifugation through a 5 % to 23 % sucrose gradient in 50 mM-Tris-HCl (pH 7.5) for

2 h at 60,000 revs./min and 15 °C. Authentic Qβ RNA was centrifuged as marker in a parallel tube. 23 fractions were collected and the ^{32}P-radioactivities determined by measuring the Cerenkov radiation. The peak fractions from the 30 S region were pooled, passed through a small column of Chelex-100 (Na^+) to remove traces of metal ions, the RNA was precipitated by ethanol, dissolved in 20 µl of 0.5 mM-EDTA and stored at -25 °C. The final yield of the full-length minus strands was 0.8 µg.

The specifically labeled minus strand preparations 4C ($\overline{HO}$CMP) and 4C (CMP) were purified as described above, but the second chromatography on Sepharose 2B and the subsequent sucrose gradient centrifugation were omitted. The [α-^{32}P]AMP incorporated in the minus stands was 0.4 pmol per nmol of [^3H]CMP (i.e. about 0.45 pmol per pmol of minus strand) incorporated in the first step.

h) Synthesis of ^{32}P-labeled plus strands using minus strands as template.

N^4-hydroxyCMP substituted minus stands were used as template for multiple rounds of replication by Qβ replicase to obtain sufficient amounts of progeny RNA. The enzyme was saturated with template at all times to reduce formation of "6 S RNA" (Flavell et al., 1975). In a typical experiment, 0.4 µg of substituted minus strands, 0.15 units each of Qβ replicase and host factor I were incubated at 37 °C in 25 µl of a solution containing 0.8 mM of each ribonucleoside triphosphate (including [α-^{32}P]ATP, specific activity 15,600 cts/min/nmol), 0.16 M-KCl, 12 mM-$MgCl_2$, 80 mM-Tris-HCl (pH 7.5), and 1 mM-EDTA. After 30 min 0.15 units each of replicase and host factor were added and incubation was continued for 30 min at 37 °C. Further amplification was obtained by doubling the amount of replicase, host factor

and by increasing the volume of the reaction mixture every 30 min, keeping the concentration of all other components constant. After 4 h of amplification, the mixture (0.15 ml) was extracted twice with 1 vol of phenol and chromatographed through a column of Sephadex G-100 (0.5 x 6 cm) containing a layer of 0.1 ml of Chelex-100 (Na^+) in 20 mM-Tris-HCl (pH 7.5), 2 mM-EDTA. The excluded material (containing 19 nmol of acid-insoluble AMP) was precipitated by ethanol. The RNA was dissolved in 100 µl of 0.5 mM-EDTA (pH 7.5), denatured in a boiling water bath for 90 sec and centrifuged through a linear sucrose density gradient as above. The fractions containing high molecular weight RNA were pooled; the yield of 30 S RNA was 6.4 µg. The RNA was further replicated <u>in vitro</u> essentially as described above. The scheme of the amplification of two independently prepared minus strand preparations is shown in the legend to Table 8.

For analysis, highly radioactive plus strands were synthesized and purified as described above, except that the labeled nucleoside triphosphate was of high specific activity (2×10^7 to 3.5×10^7 cts/min/nmol).

i) Purification of plus strands

A mixture of labeled plus and minus strands (about 5 µg) was denatured in 0.5 ml of 0.5 mM-EDTA in the presence of an excess of unlabeled Qβ RNA (80 µg). Following the addition of 20 x SSC to give a final concentration of 0.5 x SSC, the mixture was annealed for 15 min at 55 °C. The single-stranded RNA (containing the bulk of the ^{32}P-labeled plus strands) was separated from the double-stranded fraction (containing all of the labeled minus strands) by cellulose chromatography as described by Franklin (1966).

j) Isolation of the ribosome binding site (for Section III)
The procedure is based on that originally described by Hindley & Staples (1969) and by Steitz (1969). The mixture of the ^{32}P-labeled plus and minus strands (present in a ratio of about 3 : 2) was mixed with cold Qβ RNA to give a total of 90 μg RNA. This was incubated with 5 OD_{260} units of ribosomes and 130 μg of unfractionated fMet-tRNA (about 170 pmol of fMet-tRNA) under the binding conditions described under "Preparation of the 70 S Qβ RNA ribosome complex", in a final volume of 100 μl. After 10 min at 37 ^{o}C the 70 S initiation complex was purified by sucrose gradient centrifugation as described before. The complex, containing about 20 μg of [^{32}P]Qβ RNA in 2 ml of buffer B, was treated with 4 μg of RNAase A for 10 min at 25 ^{o}C and chromatographed through a 1.5 x 30 cm column of Sephadex G-200, equilibrated with 10 mM-Tris-HCl (pH 7.5), 10 mM-$MgCl_2$, 50 mM-NH_4Cl and 0.5 mM-dithiothreitol. The excluded material, consisting of ribosomes and bound [^{32}P]Qβ RNA fragments, was located by Cerenkov radiation and pooled. After incubation with pronase (0.5 mg/ml) in the presence of 0.5 % SDS and 25 mM-EDTA for 15 min at 37 ^{o}C, the RNA was extracted twice with an equal volume of phenol. After ethanol precipitation the RNA was washed twice with 66 % ethanol and dissolved in 20 μl of 0.5 mM-EDTA (pH 7.5). After denaturation in a boiling water bath for 90 seconds the sample was chilled, mixed with 5 μl of 40 % sucrose, containing some bromophenol blue, and placed into a slot (2 x 10 mm) of a 20 % polyacrylamide gel slab (2 x 200 x 400 mm), prepared according to Weber et al. (1972). The gel was run at 800 V for 24 h and autoradiographed. Gel segments corresponding to the bands were crushed, eluted with 2 ml of 20 mM-Tris-HCl (pH 7.5), 0.8 M-NaCl, 2 mM-EDTA containing 50 μg of yeast carrier RNA. The RNA was precipitated with ethanol and analyzed by 2-dimensional electrophoresis after digestion with pancreatic RNAase or T_1 RNAase as described below.

k) Isolation of the replicase S binding site

The preparation were carried out exactly as described by Weber et al. (1972).

l) Two-dimensional polyacrylamide gel electrophoresis

Two-dimensional polyacrylamide gel electrophoresis separation T_1 oligonucleotides was carried out as described by De Wachter & Fiers (1972).

FOOTNOTES

SSC is 0.15 M-NaCl, 0.015 M-sodium citrate, pH 7.0.

$\overline{HO}C$, $\overline{HO}CMP$ is N^4-hydroxyCMP.

REFERENCES

1 Bann, R.A., Hilbers, C.W., van Charldrop, R., van Leedam, E., van Knippenberg, P.H. & Bosch, L. (1977). Proc.Nat. Acad. Sci., U.S.A. 74, 1028-1031

2 Bear, O.G., Ng, R., van Derveer, D., Johnson, N.D., Thomas, G., Schleich, T. & Noller, H.F. (1976). Proc. Nat. Acad. Sci., U.S.A. 73, 1824-1828.

3 Benne, R., Arentzen, R., & Voorma, H.O. (1972). Biochim. Biophys. Acta 269, 304-310.

4 Billeter, M.A., Parsons, J.T. & Coffin, J.M. (1974). Proc. Nat. Acad. Sci., U.S.A. 71, 3560-3564.

5 Bollen, A., Heimark, R.L., Cozzone, A., Traut, R.R., Hershey, J.W. & Kahan, L. (1975) J. Biol. Chem. 250, 4310-4314.

6 Chen, C.S. & Nakamoto, T. (1978) Proc. Nat. Acad. Sci., U.S.A. 75, 167-171.

7 Czernilofsky, A.P., Kurland, C.G. & Stöffler, G. (1975) FEBS Lett. 58, 281-284.

8 Dahlberg, A.E. (1974) J. Biol. Chem. 249, 7673-7678.

9 Dahlberg, A.E. & Dahlberg, J.E. (1975) Proc. Nat. Acad. Sci., U.S.A. 72, 2940-2944.

10 de Graaf, F.K., Niekus, H.G.D. & Klootwijk, J. (1973). FEBS Lett. 161-165.

11 Domingo, E., Flavell, R.A. & Weissmann, C. (1976). Gene, 1, 3-25.

12 Draper, D.E., & von Hippel, P.H. (1976). ICN-UCLA Symp. Mol. & Cell. Biol. 5, 421-426.

13 Dube, S.K., Marcker, K.A., Clark, B.F.C. & Cory, S. (1968). Nature (London), 218, 232-233.

14 Dunn, J.J., Buzash-Pollert, E. & Studier, F.W. (1978). Proc. Nat. Acad. Sci., U.S.A. 75, 2741-2745.

15 Feix, G., Pollet, R. & Weissmann, C. (1968). Proc. Nat. Acad. Sci., U.S.A. 59, 145-152.

16 Fiers, W., Contreras, R., Duerinck, F., Haegeman, G., Merregaert, J., Min Jou, W., Raeymakers, A., Volckaert, G., Ysebaert, M., Van de Kerckhove, J., Nolf, F. & van Montagu, M. (1975). Nature (London), 256, 273-278.

17 Fiers, W., Contreras, R., Duerinck, F., Haegeman, G., Iserentant, D., Merregaert, J., Min Jou, W., Molemans, F., Raeymakers, A., van der Berghe, A., Volckaert, G. & Ysebaert, M. (1976). Nature (London) 260, 500-507.

18 Flavell, R.A., Sabo, D.L., Bandle, E.F. & Weissmann, C. (1974). J. Mol. Biol. 89, 255-272.

19 Flavell, R.A., Sabo, D.L., Bandle, E.F. & Weissmann, C. (1975). Proc. Nat. Acad. Sci., U.S.A. 72, 357-371.

20 Franklin, R.M. (1966). Proc. Nat. Acad. Sci., U.S.A. 55, 1504-1511.

21 Fukami, H. & Imahori, K. (1971). Proc. Nat. Acad. Sci., U.S.A. 68, 570-573.

22 Givol, D., Weinstein, Y., Govecki, M. & Milchek, M. (1970). Biochem. Biophys. Res. Commun. 38, 825-830.

23 Goldberg, M.L. & Steitz, J.A. (1974). Biochemistry, 13, 2123-2129.

24 Gralla, J. & Crothers, D.M. (1973). J. Mol. Biol. 73, 497-511.

25 Greenshpan, H. & Revel, M. (1969). Nature (London) 224, 331-335.

26 Grosjean, H., Söll, D.G. & Crothers, D.M. (1976). J.Mol. Biol. 103, 499-519.

27 Grosjean, H.J., de Henau, S. & Crothers, D.M. (1978). Proc.Nat.Acad.Sci., U.S.A. 75, 610-614.

28 Gualerzi, C., Risuleo, G. & Pon, C.L. (1977). Biochemistry 16, 1684-1689.

29 Hawley, D.A., Slobin, L.I. & Wahba, A.J. (1974). Biochem. Biophys. Res. Commun. 61, 544-550.

30 Held, W.A., Gette, W.R. & Nomura, M. (1974). Biochemistry 13, 2115-2122.

31 Hermoso, J.M. & Szer, W. (1974). Proc. Nat. Acad. Sci., U.S.A. 71, 4708-4712.

32 Hindley, J. & Staples, D.H. (1969). Nature (London) 224, 964-967.

33 Infante, A.A. & Baierlein, R. (1971). Proc. Nat. Acad. Sci., U.S.A. 73, 767-770.

34 Inoue, H., Pollack, Y. & Petre, J. (1974). Eur. J. Biochem. 45, 109-117.

35 Isono, K. & Isono, S. (1976). Proc. Nat. Acad. Sci., U.S.A. 73, 767-770

36 Iwasaki, K., Sabol, S., Wahba, A.J. & Ochoa, S. (1968). Arch. Biochem. Biophys. 125, 542-547.

37 Jay, G. & Kaempfer, R. (1975). J. Biol. Chem. 250, 5472-5478.

38 Kenner, R.A. (1973). Biochem. Biophys. Res. Commun. 51, 932-938.

39 Kolakofsky, D., Billeter, M.A., Weber, H. & Weissmann, C. (1973). J. Mol. Biol. 76, 271-284.

40 Langhrea, M. & Moore, P.B. (1978). J.Mol.Biol. 121, 411-430.

41 Leffler, S. & Szer, W. (1973). Proc. Nat. Acad. Sci., U.S.A., 70, 2364-2368.

42 Legon, S., Model, P. & Robertson, H.D. (1977). Proc. Nat. Acad. Sci., U.S.A. 74, 2692-2696.

43 Lodish, H.F. (1970a). Nature (London). 224, 705-707.

44 Lodish, H.F. (1970b). J. Mol. Biol. 50, 689-702.

45 Lodish, H.F. (1975). In RNA Phages (Zinder, N.D., ed.), pp. 301-318, Cold Spring Harbor Laboratory, New York.

46 Lucas-Lenard, J. & Lipmann, F. (1971). Annu. Rev. Biochem. 40, 409-448.

47 Maxam, A.M. & Gilbert, W. (1977). Proc. Nat. Acad. Sci., U.S.A. 74, 560-564.

48 Meyer, F., Weber, H. & Weissmann, C. (1976). Experientia, 32, 804.

49 Miller, M.J. & Wahba, A.J. (1973). J. Biol. Chem. 249, 3808-3813.

50 Noll, M. & Noll, H. (1974). J. Mol. Biol. 89, 477-494.

51 Pollet, R., Knolle, P. & Weissmann, C. (1967). Proc. Nat. Acad. Sci., U.S.A. 58, 766-773.

52 Ptashne, M., Backman, K., Humayun, M.Z., Jeffrey, A., Maurer, R., Meyer, B. & Sauer, R.T. (1976). Science 194, 156-161.

53 Rensing, U.F.E. & Schoenmakers, J.G.G. (1973). Eur. J. Biochem. 33, 8-18.

54 Revel, M. & Greenshpan, H. (1970). Eur. J. Biochem. 16, 117-122.

55 Sabo, D.L., Domingo, E., Bandle, E.F., Flavell, R.A. & Weissmann, C. (1977). J. Mol. Biol. 112, 235-252.

56 Sanger, F. & Brownlee, G.G. (1967). In Methods in Enzymology (Grossman, L. & Moldave, K., eds.), vol. 12 A, pp. 361-381, Academic Press, New York.

57 Schwartz, J.H., Iglewski, W.J. & Franklin, R.M. (1969). J. Biol. Chem. 244, 736-743.

58 Shine, J. & Dalgarno, L. (1974). Proc. Nat. Acad. Sci., U.S.A. (1974) 71, 1342-1346.

59 Sprague, K.U., Steitz, J.A., Grenlex, R.M. & Stocking, C.E. (1977). Nature (London) 267, 462-465.

60 Staples, D.H., Hindley, J., Billeter, M.A. & Weissmann, C., (1971). Nature New Biol. 234, 202-204.

61 Steitz, J.A. (1969). Nature (London) 224, 957-964.

62 Steitz, J.A. (1972). Nature New Biol. 236, 71-75.

63 Steitz, J.A. (1973a). J. Mol. Biol. 73, 1-16.

64 Steitz, J.A. (1973b). Proc. Nat. Acad. Sci., U.S.A. 70, 2605-2609.

65 Steitz, J.A. (1975). In RNA Phages (Zinder, N.D., ed.), pp. 319-352, Cold Spring Harbor Laboratory, New York.

66 Steitz, J.A. & Jakes, K. (1975). Proc. Nat. Acad. Sci., U.S.A. 72, 4734-4738.

67 Steitz, J.A., Sprague, K.U., Steege, D.A., Yuan, R.C., Laughrea, M., Moore, P.B. & Wahba, A.J. (1977b) In Nucleic Acid-Protein Recognition (Vogel, H.J., ed.), Academic Press, Inc. New York.

68 Steitz, J.A. & Steege, D.A. (1977). J. Mol. Biol. 114, 545-558.

69 Steitz, J.A., Wahba, A.J., Laughrea, M. & Moore, P.B. (1977). Nucleic Acids Res. 4, 1-15.

70 Subramanian, A.R., Davis, B.D. & Beller, R.J. (1969). Cold Spring Harbor Symp. Quant. Biol. 34, 223-230.

71 Szer, W., Hermoso, J.M. & Boublik, M. (1976). Biochem. Biophys. Res. Commun. 70, 957-964.

72 Szer, W. & Leffler, S. (1974). Proc. Nat. Acad. Sci., U.S.A., 71, 3611-3615.

73 Taniguchi, T. & Weissmann, C. (1978). J. Mol. Biol. 118, 533-565.

74 Thach, R.E., Sundararajan, T.A., Dewey, K.F., Brown, J.C. & Doty, P. (1966). Cold Spring Harbor Symp. Quant. Biol. 31, 85-97.

75 Tinoco, I., Jr., Borer, P.N., Dengler, B., Levine, M.D., Uhlenbeck, O.C., Crothers, D.M. & Gralla, J. (1973). Nature New Biol. 246, 40-41.

76 Uhlenbeck, O.C., Baller, J.& Doty, P. (1970). Nature (London) 225, 508-510.

77 van Dieijen, G., van Knippenberg, P.H. & van Duin, J. (1976). Eur. J. Biochem. 64, 511-518.

78 van Dieijen, G., van Knippenberg, P.H., van Duin, J., Koekman, B. & Pouwels, P.H. (1977). Molec. Gen. Genet. 153, 75-80.

79 van Duin, J., Kurland, C.G., Dondon, J. & Grunberg-Manago, M. (1975). FEBS Lett. 59, 287-290.

80 van Duin, J.& van Knippenberg, P.H. (1974). J. Mol. Biol. 84, 185-195.

81 van Knippenberg, P.H., Hooykaas, P.J.J. & van Duin, J. (1974). FEBS Lett. 41, 323-326.

82 Vermeer, C., de Kievit, R.J., van Alphen, W.J. & Bosch, L. (1973). FEBS Lett. 31, 273-276.

83 Volckaert, G., Min Jou, W. & Fiers, W. (1976). Anal.Biochem. 72, 433-446.

84 Wahba, A.J., Miller, M.J., Niveleau, A., Landers, T.A., Carmichael, G.G., Weber, K., Hawley, D.A. & Slobin, L.I. (1974). J. Biol. Chem. 249, 3314-3316.

85 Walz, A., Pirrotta, V. & Ineichen, K. (1976). Nature (London) 262, 665-669.

86 Weber, H., Billeter, M., Kahane, S., Hindley, J., Porter, A. & Weissmann, C. (1972). Nature New Biol. 237, 166-170.

87 Weissmann, C., Colthart, L. & Libonati, M. (1968). Biochemistry, 7, 865-874.

88 Weissmann, C., Billeter, M.A., Goodman, H.M.,Hindley, J. & Weber, H. (1973). Annu. Rev. Biochem. 42, 303-328.

89 Yokota, T., Arai, K. & Kaziro, Y. (1977). J. Biochem. (Tokyo). 82, 1485-1489.

Curriculum Vitae

Tadatsugu Taniguchi

Born: 1. Jan. 1948 in Wakayama (Japan)

Citizenship: Japanese

1954 - 1960	Elementary School (in Wakayama)
1966 - 1963	Middle School (in Wakayama)
1963 - 1966	Taikyu Senior High School (in Wakayama)
1966 - 1971	Tokyo University of Education (B. Sci. in Botany)
1971 - 1974	Graduate School, Tokyo University of Education (Plant Biochemistry) (During this period, I was at University of Naples, Italy, as a research fellow from 1972 to 1974.)
1974 - 1978	Research Assistant at the Institut für Molekularbiologie I, Universität Zürich.

I enrolled at the University of Zürich in 1976. The present Dissertation has been completed under the supervision of Prof. Dr. C. Weissmann at the Institut für Molekularbiologie I, Universität Zürich.

At the University of Zürich, I have attended lectures given by Prof. Dr. C. Weissmann and Prof. Dr. M. Billeter.